IDENTIFICATION AND ANALYSIS OF THE VIRULENCE FACTORS IN *Serratia entomophila* CAUSING AMBER DISEASE TO THE GRASS GRUB *Costelytra zealandica.* A MOLECULAR GENETICS APPROACH.

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A mis padres: Carlos y Ernestina

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A Javier

Quienes con su cariño, su comprensión,

su apoyo y sus consejos han iluminado mi camino

En el campo de la observación, la suerte favorece
solo a la mente que esta preparada.

Luis Pasteur

In the fields of observation, chance favors
only the mind that is prepared.

Luis Pasteur

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ABSTRACT

Amber disease caused by *Serratia entomophila* to larvae of *Costelytra zealandica* (Coleoptera: Scarabaeidae), is characterized by the production of two symptoms: anti-feeding effect (AFE) and amber coloration (AC). This study was aimed to identify and characterize the virulence factors involved in the disease. Three factors were identified: i) MRE-HA fimbriae; ii) an extracellular protease and, iii) an anti-feeding toxin.

i) Fimbriae type 1, 3 and MRE-HA were identified and characterized in *S. entomophila* by haemagglutination tests and electron microscopy. Analysis of nonpathogenic mutants suggested that the MRE-HA fimbriae were associated with pathogenicity.

ii) The locus coding for the extracellular protease of *S. entomophila* was identified and cloned. Examination and complementation assays of pathogenic and nonpathogenic strains showed that the protease is not directly involved, but it might potentiate the disease. It was suggested that the protease might be linked with pathogenicity by a common regulator factor.

iii) A locus named *amb2* was identified, isolated and cloned. Genetic evidence and complementation assays with nonpathogenic mutants demonstrated that *amb2* is responsible for the AFE. SDS-PAGE analysis of the *amb2* gene products expressed in minicells showed the synthesis of two proteins of 21 and 25 kDa, named AnfA and AnfB. The genes encoding these proteins were mapped by deletion analysis and *lacZ*-gene fusions. DNA sequencing of the *anfA* gene revealed that another protein of ~12 kDa ($AnfA_2$) was also encoded by *amb2*. Consensus sequences with homology to the binding sites of the bacterial regulators CAP, Fur and ToxR were identified in the promoter regions. Homology of 50% was found between a hydrophobic motif of the δ-endotoxin of *Bacillus thuringiensis* and $AnfA_1$. The results suggest that $AnfA_1$, $AnfA_2$ and AnfB might be subunits of a toxin causing the AFE.

It was concluded that virulence determinants in *S. entomophila* including the MRE-HA fimbriae, the extracellular protease and the anti-feeding toxin act in collaboration to produce amber disease.

CHAPTER I

GENERAL INTRODUCTION

A major insect pest in New Zealand pastures is the grass grub *Costelytra zealandica* (White) (Coleoptera: Scarabaeidae). The larva feeds on the young roots of pasture causing severe losses in the grass yield (Chapman, 1990).

Since last century, several attempts have been made to control the grass grub infestations (Hoy, 1965). As with other insect pests, the most common method used has been the application of chemical insecticides. However, because of harmful residues in the environment, the development of resistance by the pest to chemicals, and high production costs, there has been an increasingly active search for alternatives to control the pest (Moore, *et al.*, 1974; Dearing, *et al.*, 1980; Fowler, 1974; Stucki, *et al.*, 1984; Cameron and Wigley, 1989). Recently a new bacterial species of the genus *Serratia* has been shown to cause a fatal disease known as "amber disease" in the grass grub *C. zealandica* (Trought, *et al.*, 1982; Stucki, *et al.*, 1984; Grimont, *et al.*, 1988).

The bacterium *Serratia entomophila* (*Enterobacteriaceae*) colonises the larval gut after ingestion from the soil (Wilson, 1988). The infection causes cessation of feeding, clearing of the gut and development of an amber coloration (Trought, *et al.*, 1982; Jackson, *et al.*, 1993). Disease symptoms are evident before invasion of the hemocoel by the bacteria (Jackson, *et al.*, 1993) which seems to occur at later stages of the disease. Death of the larvae appears to be caused by septicemia after several weeks of infection.

The bacteria have been used in field trials and shown high levels of pathogenicity to the grub, resulting in a reduction of the pest population of up to 60 % (Jackson, *et al.*, 1986). *S. entomophila* is highly specific, appearing to be harmless to other insects and animals (Jackson, *et al.*, 1992). *S. entomophila* has been developed as a commercial bioinsecticide against larvae of *C. zealandica* (Jackson, *et al.*, 1992).

As part of a general research program on *S. entomophila*, several studies have focused on the search for pathogenic determinants of amber disease and the identification of genetic loci involved in bacterial pathogenicity. Ultimately it should

be possible to improve the efficiency of the bacteria to increase virulence and extend the host range of *S. entomophila* to other insect pests.

1) Bacterial pathogenicity

A complex array of molecular mechanisms have evolved for successful multiplication and establishment of a pathogen within the host.

Most studies on pathogenicity have been done on microorganisms affecting vertebrates. However, it is being recognized that bacterial pathogens usually share similar mechanisms to colonize and reproduce inside the host, causing a disease (Finlay and Falkow, 1989). Therefore, similar or analogous mechanisms of pathogenicity might be present in invertebrate and vertebrate pathogens. General mechanisms such as colonization, fimbriae and production of toxins are briefly described below.

1.1) Colonization and fimbriae.

Colonization of a pathogen in specific sites of the host requires the attachment of the bacteria to cells surfaces. This is conducive to the establishment and reproduction of the pathogen within the host. Attachment to the epithelial cells of the intestine is, for some enterobacteria, an essential step to further invasion of inner parts of the host. Attachment or adhesion is usually mediated by the interaction of a protein located on the surface of bacteria and specific carbohydrates situated on the eukaryotic cell membrane. The former is called the adhesin and the latter the receptor.

Species of *Enterobacteriaceae* present protein appendages on the cell surface called fimbriae or pili. Adhesins are generally integrated in the fimbrial structure of pathogenic bacteria; therefore, fimbriae have been associated with virulence. However, nonfimbrial adhesins have also been reported and non pathogenic strains may also produce pili (Clegg and Gerlach, 1987; Finlay and Falkow, 1989). Therefore, the sole presence of these structures do not determine pathogenicity and their role in this phenomenon has been difficult to discern. Nevertheless, many studies at the molecular level, mainly carried out with *E. coli*, have found that specific fimbriae and adhesins are involved in pathogenicity.

Bacterial fimbriae have been classified on the basis of haemagglutination properties. Type 1 fimbriae have the ability to bind to D-mannose residues located on eukaryotic cells and so they agglutinate erythrocytes (Old, 1972). Agglutination is inhibited in the presence of that carbohydrate; therefore showing mannose sensitive haemagglutination (MS-HA). The role of type 1 fimbriae in pathogenicity is unclear. However, they have been associated with infection of the urinary tract by some strains of *E. coli* (Aronson, *et al.*, 1979; Hultgren, *et al.*, 1985), *Serratia marcescens* (Yamamoto, *et al.*, 1985) and *Klebsiella pneumoniae* (Mayan, *et al.*, 1985).

Mannose-resistant haemagglutination fimbriae (MR-HA) have been frequently associated with bacterial virulence. These types of pili include the P fimbriae of *E. coli* involved in acute infant pyelonephritis and cystitis (Hagberg, *et al.*, 1981). Adhesins in these pili do not recognize mannose as eukaryotic receptor. Instead, they attach to the disaccharide α-Gal(1-4)ß-Gal (Hoschützki *et al.*, 1989; Korhonen, *et al.*, 1982b). Adhesins with affinity to different receptors have also been published (Finlay and Falkow, 1989; Korhonen, *et al.*, 1984).

Enterotoxigenic *E. coli* (ETEC) also presents mannose-resistant fimbriae that mediate adhesion to the mucosa of the small intestine in humans. Their adhesins have been termed colonization factor antigens (CFAs), of which there are several types (Evans, *et al.*, 1977; Smyth and Smith, 1992).

Other pili with different characteristics, have been identified. These include the type 2, 3 and 4 fimbriae. The type 2 seems to be a non-adhesive variant of the type 1 and has been described only in *Salmonella* spp. (Old and Payne, 1971). Type 3 fimbriae have been described in some strains of *Enterobacteriaceae* including *Klebsiella* (Old, *et al.*, 1985), *Salmonella* (Adegbola, *et al.*, 1983), *Yersinia* (Old and Adegbola, 1984) and *Serratia* (Old, *et al.*, 1983). The special characteristic of these pili is that they agglutinate erythrocytes only when they have been treated with tannic acid. Another class of pili, the type 4, has been found in *Pseudomonas aeruginosa*, *Neisseria gonorrhoeae*, *Vibrio cholerae* and others (Paranchych and Frost, 1988). These fimbriae contain a pilin subunit with a methylated phenylalanine at its amino terminus. The role of type 4 fimbriae in pathogenicity is unknown.

The presence of a complex array of different pili types and their expression in a large number of bacteria, pathogenic and nonpathogenic, suggest that these appendages have an important role in the life cycle of many microorganisms.

Fimbriae in pathogenic bacteria, may confer important advantages for survival and reproduction under different environments in the host. Yet, these appendages represent only one of the many factors involved in pathogenicity.

1.2) Production of toxins.

Another important virulence determinant is the production of bacterial toxins that interfere in the normal function of the host.

Middlebrook and Dorland (1984) classified toxins into two groups: membrane damaging and intracellular acting toxins. The first group refers to cytolytic enzymes which interact with plasma membranes. In this class, phospholipases are known for their activities against epithelial cells, erythrocytes (Yamakawa and Ohsaka, 1977) and haemocytes of insect hemolymph (Lysenko, 1981). Proteases and chitinases are considered also important toxins for insects. Proteases attack haemocytes (Madziera-Borusiewicz and Lysenko, 1971) and chitinases have been shown toxic when injected into larvae hemolymph (Lysenko, 1976). Phospholipases, proteases and chitinases apparently depress the insect immune system and, as a consequence, contribute to virulence of bacteria.

The second group of toxins, involving intracellular activity, includes cholera toxin (CT) from *Vibrio cholerae* (Gill, 1976; Cassel and Pfeuffer, 1978), the heat labile enterotoxin (LT) from *E. coli* (Spangler, 1992), diphtheria toxin from *Corynebacterium diphtheriae* (Collier, 1975), exotoxin A from *Pseudomonas aeruginosa* (Vasil, *et al.*, 1977) and Shiga toxin from *Shigella dysenteriae* (Olsnes, *et al.*, 1981). All these toxin proteins share a general structure of two-components: the A component (α-chain) confers the biological activity and the B component (ß-chain) mediates the receptor binding to specific eukaryotic cells. With the exception of LT, the other toxins are synthesized in proenzyme form which is inactive and need to be proteolytically processed to release the enzymatic A fragment (Middlebrook and Dorland, 1984). There are also similarities among enzymatic activities of these bacterial toxins. Some of them share a general ADP-ribosyltransferase activity, which produce alterations in biosynthetic and/or metabolic functions of eukaryotic cells. Diphtheria and *Pseudomonas* A toxin inactivate cytoplasmic proteins causing inhibition of protein synthesis (Collier, 1975; Iglewski and Kabat, 1975; Iglewski, *et al.*, 1977). CT and LT stimulate adenylate cyclase activity causing high levels of cyclic AMP (Cassel and Pfeuffer, 1978; Gill and Meren, 1978). The final effect of

these enterotoxins, is an extreme loss of fluid and electrolytes in the host that may lead to death.

The insect δ-endotoxins from *Bacillus thuringiensis* (Bt), apparently share also the two-component toxin system (Spangler, 1992). Although the δ-endotoxins function as an independent polypeptide unit, one region of the protein mediates receptor binding and the other is responsible for the biological activity (Höfte and Whiteley, 1989; Li, *et al.*, 1991). The crystal proteins of the δ-endotoxins dissolve in the larval midgut and are also proteolytically processed into an active form (Lecadet and Dedonder, 1967; Haider, *et al.*, 1986). Unlike vertebrate enterotoxins which operate inside the cells, Bt δ-endotoxins act on the midgut epithelial cells of insects, forming pores in the plasma membrane (Knowles and Ellar, 1987; Höfte and Whiteley, 1989; Li, *et al.*, 1991). Consequent symptoms of toxemia in lepidopteran and dipteran larvae are paralysis of the gut and changes in gut permeability. This leads to cessation of feeding followed by swelling and lysis of the midgut epithelial cells (Aronson, *et al.*, 1986). Subsequent death of the insect occurs through starvation and septicaemia (Li, *et al.*, 1991).

2) Regulation of pathogenicity

The regulation of the genetic information of bacteria seems to be very complex and is organized in regulons. These are defined as groups of genes under the control of a common regulator. Virulence genes appear to be regulated, rather than constitutive, and the signal to control their expression is provided by the environment. Regulons controlling virulence genes have been described in several pathogenic species. One of the best studied regulatory systems mainly involved with pathogenicity is the *toxRS* locus of *V. cholerae.* These global regulators control the expression of the *ctxAB* operon of cholera toxin, a toxin co-regulated pilus (Tcp), an accessory colonization factor (Acf) and two major outer membrane proteins, OmpU and OmpT. Also, seventeen genes denominated *tag* are activated by ToxR (Peterson and Mekalanos, 1988; DiRita and Mekalanos, 1989; Taylor, *et al.*, 1987). Most of the *tag* genes appear to be involved in Tcp and Acf biogenesis.

ToxR is a transcriptional activator that specifically binds to a tandemly repeated sequence in the promoter of the *ctxAB* operon and activates its expression (Miller, *et al.*, 1987). Yet, ToxR requires the product of the *toxS* gene to accomplish this activation. In addition, members of the ToxR regulon may be also controlled by

other regulator named ToxT (DiRita, *et al.*, 1991). Some virulence genes are directly activated by ToxR, while others require the intermediate regulator ToxT.

Based on this complex control system, a model of a regulatory cascade in *V. cholerae* has been proposed (DiRita, *et al.*, 1991; 1992). The product ToxR controls the expression of the intermediate regulator ToxT and this protein then activates expression of other genes in the regulon. Expression of the *toxRS*-dependent genes is modulated by environmental factors such as aeration, osmolarity, pH, temperature and presence of some aminoacids (Betley, *et al.*, 1986; Miller and Mekalanos, 1988; Parsot and Mekalanos, 1990).

Similar regulatory systems have been reported in other pathogenic bacteria. The *vir* locus of *Bordetella pertussis*, the causative agent of whooping cough in humans, is a positive inducer of many virulence genes forming a regulon. This regulon is composed of the genes coding for pertussis toxin, adenylate cyclase, hemolysin, pili, filamentous haemagglutinin and a dermonecrotic toxin (Weiss and Hewlett, 1986). The *vir* locus responds to environmental stimuli such as temperature and the concentration of nicotinic acid and $MgSo_4$ (McPheat, *et al.*, 1983; Melton and Weiss, 1989) and modulates expression of virulence genes.

The *algD* gene of *P. aeruginosa* seems to be also a key regulator in virulent strains and is subject to environmental control through a network of regulatory genes. Osmolarity, oxygen tension and nitrogen starvation are environmental factors affecting regulation (DeVault, *et al.*, 1989).

The expression of virulence factors in bacteria seems to be dependent on a complex regulatory machinery able to sense changes in the environment. A well characterized example of environmental sense regulation is the "two component" EnvZ/OmpR system in *E. coli* and *S. typhimurium*. EnvZ and OmpR proteins are encoded by the *ompB* operon (Bachmann, 1990; Sanderson and Hurley, 1987). In this regulatory system, one protein (EnvZ) is a sensor of parameters (such as osmolarity) in the environment. EnvZ transmits information to the regulator protein by covalent modification (phosphorylation). The regulator (OmpR) is a DNA binding protein which may activate and repress transcription of the porin proteins OmpC and OmpF respectively in conditions of high osmolarity. Conversely, OmpR may repress OmpC and activate OmpF when bacteria are in an external environment, where low osmolarity conditions prevail. The products of the *ompB* operon also regulate other

S. typhimurium genes (Dorman, *et al.*, 1989) and seem to be involved in expression of virulence determinants (Chatfield, *et al.*, 1991; Bernardini, *et al.*, 1990).

In addition to a complex regulation, infection and disease are usually determined by multiple factors. Therefore, the study of bacterial pathogenicity requires an integrated analysis of the pathogenic determinants and their regulatory system(s).

3) *Serratia* spp. as insect pathogens

Infection to insects caused by *Serratia* spp. have been frequently reported (Grimont *et al.*, 1979), mainly those caused by *S. marcescens* (Steinhaus, 1959; Podgwaite and Cosenza, 1976ab; Slatten and Larson, 1967; Poprawski and Yule, 1990). Other *Serratia* species usually associated with insects are *S. liquefaciens* and *S. marinorubra* (Grimont, *et al.*, 1979).

The general mechanism of virulence in *Serratia* spp. remains unclear (Flyg and Xanthopoulos, 1983). However, some of these microorganisms have been recognized as invasive pathogens (Steinhaus, 1959; Podgwaite and Cosenza, 1976b). Once the bacteria are in the gut of the insect host, they break through the intestine membrane and invade the hemocoel. The bacterium attacks the insect immune system and reproduces itself, finally causing death by septicemia.

Attempts to characterize infections caused by *Serratia* spp. are limited. Chitinases (Podwaite and Cosenza, 1976b; Flyg and Boman, 1988; Glare and Jackson, 1990; Corbett, *et al.*, 1990; Glare, *et al.*, 1992), lecithinases (Steinhaus, 1959) and proteases (Flyg and Boman, op. cit.; Kaska, 1976) have been suggested as pathogenic determinants due to the invasive character of *Serratia* spp. infections. It has been speculated that these enzymes might collaborate on the degradation of the protective wall on the gut membrane, therefore enabling the bacteria to get access to the insect hemocoel. The genes coding chitinases (Fuchs, *et al.*, 1986) and proteases (Nakahama, *et al.*, 1986) of *S. marcescens* have been identified and cloned. However, the role of these enzymes in pathogenicity has not been defined.

Two chitinases are apparently synthesized by *S. entomophila* (Corbett, 1990). One of them has been cloned and partially characterized (Shum, 1992). Although no clear evidence has been so far presented on the role of *S. entomophila* chitinase in

amber disease, invasion of bacteria into the hemocoel requires the degradation of the gut membrane, which is in part lined with chitin. Therefore, chitinases may be implicated in the final phase of the disease (Shum, 1992).

Adhesion of the bacteria to insect cells seems to be a factor influencing *S. marcescens* virulence (Chadwick, *et al.*, 1990) and adhesion of *S. entomophila* mediated by bacterial fimbriae to the *C. zealandica* larvae gut, appears to be important for the development of amber disease (Wilson, 1988; Jackson, *et al.*, 1993; Upadhyaya, *et al.*, 1992). Several *S. entomophila* mutants were created by transposon (Tn*phoA*[1]) mutagenesis (Upadhyaya, *et al.*, 1992). The mutants UC21 and UC24 failed to produce amber disease. Analysis of the mutant UC21 revealed that the insertion of the Tn*phoA* disrupted a genetic locus apparently implicated in the biogenesis of *S. entomophila* fimbriae. This locus, named *amb1*, has been cloned and its association in amber disease has been discussed (Upadhyaya, *et al.*, 1992).

Amber disease caused by *S. entomophila* to grass grub after ingestion is characterized by an anti-feeding effect and the development of amber coloration after 3-5 days of infection (Jackson, *et al.*, 1993; Fig.1.1). These symptoms appear without invasion of the hemocoel, unlike other insect *Serratia* spp, which produce invasion and death in a short period of time (Steinhaus, 1959; Podgwaite and Cosenza, 1976b). This special feature suggests the production of an anti-feeding toxin by *S. entomophila*, essential for the development of amber disease. The insect dies usually between one and three months after infection.

The molecular and genetic factors determining the pathogenicity of *S. entomophila* remain unclear. The identification and characterization of the virulence factors is essential for the understanding of amber disease. Moreover, the cloning and analysis of the virulence genes, offer the molecular basis for the development of improved biological control systems for *C. zealandica* or similar insect pests.

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[1] A transposon is a DNA sequence able to replicate and insert one copy at a new location in a genome (Lewin, 1987), causing a mutation. Tn*phoA* contains sequences of a reporter gene encoding alkaline phosphatase. These sequences may insert into a gene generating fusions to the protein product of that gene (Manoil and Beckwith, 1985).

Fig. 1.1. Field collected larvae of *C. zealandica*. Healthy larvae (A); larvae infected with *S. entomophila* (B). Amber coloration is evident (B).

OBJECTIVES:

GENERAL AIM:

To identify and characterize the virulence factors involved in the amber disease caused by *S. entomophila* infecting larvae of *C. zealandica.*

SPECIFIC AIMS:

1) Fimbriae

a) Identification and characterization of fimbriae in *S. entomophila.*

b) Identification of the type of fimbriae associated with pathogenicity.

c) Development of *in vitro* strategies to assess adhesion properties of *S.entomophila* associated with fimbriae and virulence.

2) Proteases

a) Identification and cloning of the protease gene of *S. entomophila.*

b) Evaluation of the role of proteases in amber disease.

3) Anti-feeding activity

a) Identification and cloning of *S. entomophila* gene(s) related with amber disease symptoms by analysis of the nonpathogenic mutant UC24.

b) Characterization of the cloned genes by complementation of nonpathogenic mutants.

c) Identification and molecular analysis of the gene products involved in amber disease by minicell protein system.

d) DNA-sequencing and analysis of sequence data.

CHAPTER II

FIMBRIAE, HAEMAGGLUTINATION PROPERTIES AND INFECTIVITY IN *Serratia entomophila*

1) INTRODUCTION

Different pathogenic species of the family *Enterobacteriaceae* are known to possess special cell surface appendages called pili or fimbriae (Clegg and Gerlach, 1987). It has been demonstrated that the fimbriae function as virulent determinants in pathogenic bacteria (Smyth and Smith, 1992; Clegg and Gerlach, 1987; O'Hanley, *et al.*, 1985; Korhonen, *et al.*, 1982a; Hagberg, *et al.*, 1981; Fader and Davis, 1980) and it is thought that adherence of the microorganisms to tissue surfaces is mediated by these structures.

The presence of fimbriae in bacteria has been associated with the property of haemagglutination. Type 1 fimbriae have been associated with mannose-sensitive haemagglutination (MS-HA), so agglutination of erythrocytes is observed in the absence of α-D-mannose but not in its presence (Old, 1972). Type 2 fimbriae, although morphologically similar to type 1 pili, do not have the haemagglutination ability (Old and Payne, 1971). Fimbriae mediating haemagglutination either in the presence or in the absence of α-D-mannose are known as mannose-resistant fimbriae (MR-HA). Several types of these fimbriae have been described (Hagberg, *et al.*, 1981; Hacker, 1990; Smyth, 1982). One of them, the type 3 fimbriae (MR/K-HA), has the special characteristic of producing haemagglutination only when the erythrocytes have been treated with tannic acid (Duguid, 1959). Another type, named mannose-resistant eluting haemagglutination fimbriae (MRE-HA), produce stable agglutination of erythrocytes only at 4°C (Evans, *et al.*, 1978; Ip, *et al.*, 1981).

Some species of bacteria possess only one type of fimbriae but other species or strains produce several kinds of them and therefore they have multiple haemagglutination activities (Adegbola and Old, 1982; Smyth and Smith, 1992; Parment, *et al.*, 1992), which makes the fimbriae characterization difficult.

Information on fimbriae of the genus *Serratia* is scarce. However, it has been shown that *Serratia* spp. have fimbriae types 1 and 3 (Duguid, 1959; Yamamoto, *et al.*, 1985; Old, *et al.*, 1983; Clegg and Gerlach, 1987; Parment, *et al.*, 1992). The

presence of MR-HA fimbriae in *S. marcescens* has also been reported (Jingushi, *et al.*, 1987; Parment, *et al.*, 1992). Important studies on correlation of fimbriae and hemagglutinating properties by Adegbola and Old (1982; Old, *et al.*, 1983) showed that *Serratia* spp. have a complex system of fimbriae types.

Previous studies on amber disease have suggested that adhesion of *S. entomophila* to the larvae gut by fimbriae is an important factor in pathogenicity (Wilson, 1988; Wilson, *et al.*, 1992; Upadhyaya, *et al.*, 1992). A genetic locus named *ambl*, together with its encoding polypeptides has been implicated in amber disease and pili production (Upadhyaya, *et al.*, 1992). This study established a correlation among three factors: i) the capacity of *S. entomophila* to cause amber disease, ii) the presence of fimbriae on the bacteria, and iii) the ability to adhere to the insect gut. However, the type of fimbriae involved has not been determined and a suitable *in vitro* method for analyzing adhesion of *S. entomophila* strains independent of insect larvae has not been developed. On the other hand, since *S. entomophila* may express several different types of fimbriae, precise correlation among these structures, pathogenicity and adhesion properties is essential for unravelling virulence factors.

2) MATERIALS AND METHODS

2.1) Bacterial strains

The bacterial strains used in this work are given in Table 2.1. The wild type *S. entomophila* strain A1MO2 was originally isolated from the gut of diseased *C. zealandica* larvae (Stucki, *et al.*, 1984). Bacteria were maintained at -80°C in LB medium containing 25% (vol/vol) glycerol. Cells were regularly grown in LB medium or on LB-agar. Antibiotics were added: 50 μg of ampicillin (Ap), 50 μg Kanamycin (Km), 30 μg gentamycin (Gm) or 15 μg of tetracycline (Tc) per ml for selection with *E. coli* or some strains of *S. entomophila*. For some tests, 100 μg/ml of Km and 30 μg of Tc were used as *S. entomophila* is highly resistant to low concentrations of antibiotics. *S. entomophila* strains were grown routinely at 30°C and *E. coli* at 37°C.

2.2) Culture media for Haemagglutination tests

Nutrient broth (NB) (No.2, Oxoid Ltd.) was used alone or supplemented with

Table 2.1. Bacterial strains used in this study

Strain	Relevant Characteristics	Reference
S. entomophila		
A1MO2	Wild-type; $Path^+$	T.A. Jackson
UC9	Clonal selection from A1MO2; Ap^r $Path^+$	H.K. Mahanty
UC7	Spontaneous Km^r mutant from A1MO2; Ap^r Km^r $Path^-$	H.K. Mahanty
UC21	Tn*phoA* mutant derivative from UC9; $Path^-$ Ap^r Km^r Gm^r	195
E. coli		
HB101	F^- *pro leu thi lacY recA hsdR hsdM*	166

10% (vol/vol) of skim milk (0.1 g/ml in H_2O) and antibiotics. Agar was added (1.5%) for bacteria grown on plates. Phosphate buffer-agar (PBA) was nutrient agar buffered at pH 7.0 with 3.6 g of KH_2PO_4 and 6.4 g of Na_2HPO_4 per litre, prepared as reported (Adegbola and Old, 1982). CFA-agar (colonization factor antigen-agar) was prepared as described (Evans, *et al.*, 1979) by mixing casamino-acids (1%, w/vol), yeast extract (0.15%), $MgSO_4$ (0.005%), $MnCl_2$ (0.0005%) and agar (2%), adjusted to pH 7.4.

2.3) Growth conditions for haemagglutination tests

Bacteria were cultured (serially in aerobic, static broths, four times with 5-days intervals) essentially as reported previously (Old and Duguid, 1970). Briefly, one colony of each strain was plated on LB-agar and grown overnight at 30°C. Bacteria were harvested and resuspended in 0.5 ml of LB. An inoculum of 200 µl of bacteria suspension, about 10^8 viable bacteria per ml, was used for cultures in 1.5 cm diameter tubes with plastic lids and 10 ml of broth. Tubes were incubated static (without agitation) and aerobically (with no artificial aeration) at 30 and 37°C. These tubes correspond to the first culture series. After 5 days of growth, 5 ml from the top of the culture was pelleted by centrifugation at low speed and used as inoculum for the next tube (second culture series). The inoculum for the second culture series was a mixture of fimbriate and non-fimbriate cells. The growth of the former is favoured in these conditions (Old and Duguid, 1970). Further culture series were prepared in a similar way. Parallel cultures were used for haemagglutination tests and electron microscopy observations.

2.4) Preparation of erythrocytes for haemagglutination tests

Fresh and tanned erythrocytes from guinea pig, horse, sheep and ox were used. Erythrocytes were washed twice in saline solution (0.85% [wt/vol] NaCl) and suspended to 7% (erythrocytes pellet vol/vol) in the same solution. Tanned erythrocytes were prepared by incubating washed red cells with 0.003% (wt/vol) tannic acid for 10 min. at 37°C as reported (Duguid, 1959).

2.5) Determination of agglutination properties and correlation with fimbriae

Haemagglutination (HA) tests were carried out as described by Old (1985). Briefly, bacteria were centrifuged and washed twice in saline solution (0.85% [wt/vol]

NaCl). Cells were suspended in the same solution to approximately $3x10^{10}$ to $5x10^{10}$ bacterial cells per ml. Dilutions of bacteria were performed and haemagglutination tests were carried out until negative results appear. Slide agglutination tests, called also "manual rocked tests" (RT) were performed by mixing equal volumes (25 µl) of bacterial cells and erythrocytes on the surface of a glass microscope slide. When agglutination was observed after manual shaking for 10 min at room temperature, the result was considered as positive.

Bacteria were analyzed by the "static settling test" (ST) basically as reported (Old, 1985) for mannose resistant eluting agglutination (MRE-HA). Settling tests were made with 20 µl of each suspension of bacteria and erythrocytes in microtitre trays in the presence of 1% (wt/vol) of α-D-mannose. After 1 hr at 4°C, results were taken. When agglutination was observed for any culture of bacteria with fresh erythrocytes of any kind in the absence but not in the presence of α-D-mannose, a mannose-sensitive haemagglutination (MS-HA) was considered to be present. When agglutination of erythrocytes of any kind was observed in the presence of α-D-mannose, a mannose-resistant haemagglutination (MR-HA) was considered positive. When a culture of bacteria agglutinated sheep or ox erythrocytes treated with tannic acid, but not fresh, in the presence of α-D-mannose, MR/K-HA was recorded.

Haemagglutination properties were correlated with a kind of fimbriae when both characteristics were observed in the same culture.

2.6) Transmission electron microscopy (TEM)

Bacterial suspensions were suspended in distilled H_20 and negatively stained with 2% of phosphotungstic acid, pH 6.5 for 1 min. One drop of the suspension was placed on a formvar coated grid. The bacteria were examined under a Jeol JEM 1200EX electron microscope at 80 kV.

3) RESULTS

3.1) Analysis of *S. entomophila* fimbriae

3.1.1) Growth conditions for fimbrial production

In order to identify the type of fimbriae that may be involved in amber disease, haemagglutination tests and electron-microscopical (TEM) examinations were

carried out with pathogenic and nonpathogenic *S. entomophila* strains.

Previous observations of wild type bacteria showed very low proportion (1-2%) of fimbriated cells in culture (Wilson, 1988; Glare and Jackson, 1990). Attempts were made to increase this proportion, since characterization of pili types in these conditions was difficult. Studies with *E. coli* (Duguid, 1959) showed that expression of the different type of fimbriae seems to be favoured in certain growing conditions. *E. coli* was found to vary between a fimbriate and a non-fimbriate phase, the former becoming predominant after several serial aerobic cultivations in broth (Old and Duguid, 1970). On the other hand, the type of pili associated with enterotoxigenic strains in *E. coli* (MR-HA) appear to be promoted by cultures on agar plates (Evans, *et al.*, 1979; Evans, *et al.*, 1978). Therefore, different culture conditions and media composition were tested. Broth and agar culture medium was used. Luria-Bertani (LB), Nutrient broth (NB), CFA (casamino acids plus trace elements) and PBA (buffered NB) were employed. In addition, culture media supplemented with beef extract and milk was tested. Static and shaking conditions were employed for all these culture media. Cells were grown at 30 and 37°C, since fimbriae in some species of *Serratia* seem to be promoted at 30°C (Adegbola and Old, 1982).

Microscopic examinations of wild type *S. entomophila* (A1MO2) showed an increment in the proportion of piliated cells in cultures grown in NB under static conditions. However, this increment was only in the order of 4%-5%. When NB was supplemented with milk, not only the proportion of pilliated cells increased to 10% but also morphologically different kinds of pili were distinguishable. Increments of 4-5% of piliated cells were observed in cultures on CFA and NB-agar supplemented with milk. Therefore, based on these observations, culture conditions were established and all the strains were grown and examined in serial and static cultures at 5 days intervals in tubes of NB broth with and without milk, at 30°C and 37°C. Cultures grown on different agar media were tested and examined as well.

3.1.2) Characterization of Hemagglutinins (HAs)

Pathogenic *S. entomophila* strains A1MO2 and UC9 were examined for their ability to produce haemagglutination. *E. coli* HB101 was used as a negative control, since it has been reported as non-piliated (Blomfield, *et al.*, 1991). Tests were done with erythrocytes from different sources by RT and ST as described in Materials and Methods. Results of these experiments are summarized in Table 2.2. Strong

Table 2.2. Hemagglutination observed in *S. entomophila*

	Type of hemagglutination[f]		
Strain	MS[a] (H)[e]	MR/K[b] (TS0)[e]	MRE[c] (HSOG)[e]
A1MO2	+++	++	+++
UC9	++	+	+++
UC21	+++	++[d]	-
UC7	+	++[d]	+++

[a] first culture series in NB and/or overnight on LB plates demonstrated by RT

[b] first culture series in NB demonstrated by ST

[c] first culture series in NB plus milk

[d] fourth culture series for tanned ox erythrocytes in NB

[e] spectrum of hemagglutination: horse (H), sheep (S), ox (O), guinea pig (G), tanned erythrocytes (T).

[f] arbitrary degrees of agglutination from - to +++

agglutination of horse erythrocytes tested by rocked test was observed in both strains, yet the effect was stronger for A1MO2 than for UC9. Haemagglutination is inhibited by the presence of α-D-mannose, so it is related with MS-HA. This feature was evident only in cells grown at 30°C; bacteria grown at 37°C were not able to show this effect. MS-HA was not observed with any other erythrocytes tested for. All the culture conditions stated above appeared to be suitable for *S. entomophila* to cause MS-HA. However cells grown in broth supplemented with milk in static conditions, presented MS-HA only after 48 hours of growth in the first serial culture. At longer times or in subsequent cultures of the series, haemagglutination of horse erythrocytes was not suppressed by mannose. This second type of agglutination was related to mannose resistant haemagglutination (MR-HA).

MR-HA was observed by ST in bacteria grown in broth supplemented with milk after 5 days of growth in the first serial culture. Subsequent cultures also showed MR-HA (Table 2.3 and Fig. 2.1). Haemagglutination was observed in the microtitre trays with the different blood types tested (Fig. 2.2). Erythrocytes from sheep (S), ox (O), horse (H) and guinea pig (G) were agglutinated by both strains tested in the absence and presence of α-D-mannose. Agglutination was stronger in the latter conditions (Fig. 2.1). This MR-HA was usually very weak and showed the property of "elution", interaction with erythrocytes more stable at 4°C (Evans, *et al.*, 1978; Ip, *et al.*, 1981). Bacteria growing in broth without milk showed MR-HA only after 5 days of the fourth serial culture.

MR/K-HA was demonstrable in both pathogenic *S. entomophila* strains by ST. This haemagglutination was detected independently of the MS-HA and MRE-HA because it is demonstrated only with erythrocytes from sheep and ox tanned erythrocytes in the presence of α-D-mannose. On the contrary, MS-HA and MRE-HA are demonstrated with fresh blood (untanned). MRK-HA was produced by cells growing in broth with no milk supplement, after 5 days of growth in the first serial culture and by cells grown overnight on LB plates, demonstrable only by ST. No haemagglutination was observed in similar conditions for fresh sheep and ox erythrocytes (Fig. 2.1).

E. coli HB101 did not show MRE-HA or MR/K-HA as was expected, but surprisingly it showed MS-HA with horse erythrocytes, demonstrated by RT when it was grown in broth supplemented with milk at 30°C in the first serial culture.

Table 2.3. Hemagglutination pattern of *S. entomophila*[a]

Strain	HA reaction[b] in absence/presence of α-D-mannose with erythrocytes tested of: sheep	T[c] sheep	guinea pig	ox	T[c] ox	horse
A1MO2	+/++	+++	++/++	++/++	+++	+++/+++
UC9	++/+++	+++	++/++	++/++	+++	++/+++
UC21	-/-	-	-/-	-/-	-	-/w
UC7	++/++	+++	+++/+++	++/+++	+++	+++/+++

[a]Pathogenic (A1MO2, UC9) and nonpathogenic strains (UC21, UC7) were grown in NB supplemented with milk at 30°C in static cultures during 5 days (firt culture series). Results of static settling tests.

[b] arbitrary degrees of agglutination from - to +++

[c]tanned erythrocytes. Test done only in presence of mannose.

w= weak

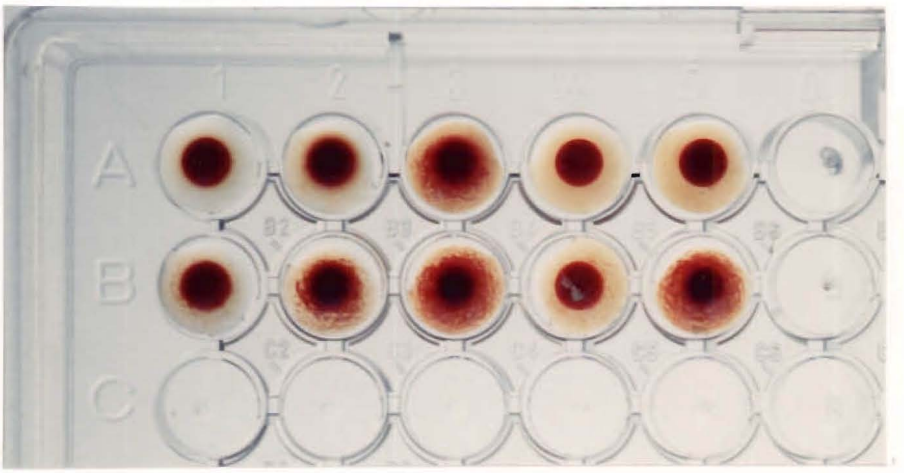


Fig. 2.1. Static settling test for mannose resistant eluting haemagglutination (MRE-HA) of *S. entomophila* A1MO2 observed in NB supplemented with milk . Bacteria were cultured serially, in static conditions at 30°C in NB (row A) and NB supplemented with milk (row B). Bacteria were mixed with erythrocytes in absence (M-) and presence (M+) of α-D-mannose and incubated at 4°C, 1 hr. No haemagglutination was observed in bacteria grown in NB without milk (A 1, A2, A4, A5). Haemagglutination observed in A3 was related with MR-K/HA. MRE-HA fimbriae were apparently stimulated in NB supplemented with milk, so haemagglutination was observed in row B. Erythrocytes from: 1) sheep, M-; 2) sheep, M+; 3) sheep (tanned), M+; 4) guinea pig, M-; 5) guinea pig, M+.

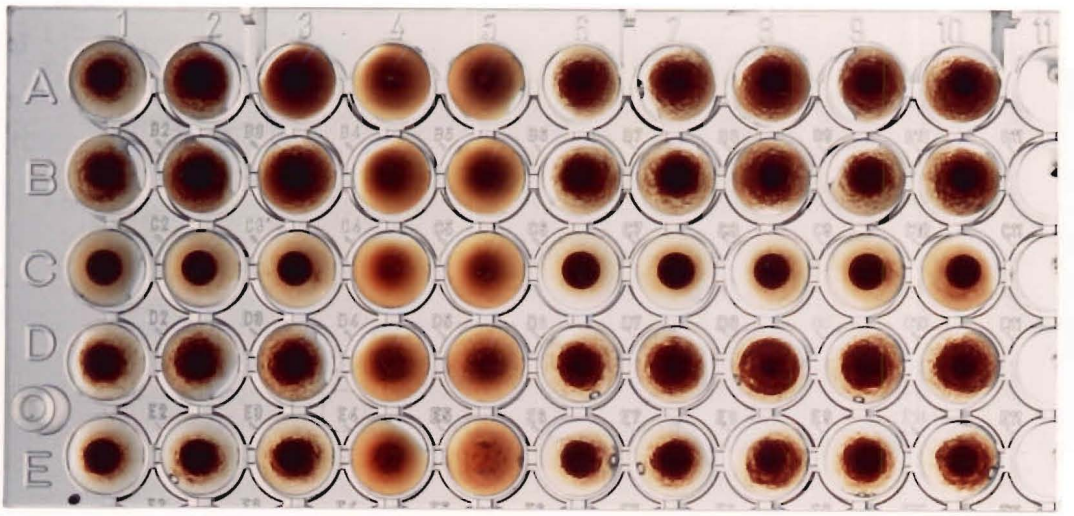


Fig. 2.2. Static settling test for mannose resistant eluting haemagglutination (MRE-HA) of *S. entomophila* pathogenic (Path$^+$) and nonpathogenic (Path$^-$) strains. Bacteria were cultured serially, in static conditions at 30°C in NB supplemented with milk and suitable antibiotics. Bacteria were mixed with erythrocytes in absence (M-) and presence (M+) of α-D-mannose and incubated at 4°C, 1 hr. Results from the first culture series after 5 days of growth are presented. No haemagglutination (C1-C9) or weak haemagglutination (C10), was observed in the nonpathogenic strain UC21. Different strains are in row: A) A1MO2, Path$^+$; B) UC9, Path$^+$; C) UC21, Path$^-$; D) UC7, Path$^-$; E) UC7/*amb2* (see Chapter IV). Different erythrocytes are in lane: 1) sheep, M-; 2) sheep, M+; 3) sheep (tanned), M+; 4) guinea pig, M-; 5) guinea pig, M+; 6) ox, M-; 7) ox, M+; 8) ox (tanned), M+; 9) horse, M-; 10) horse, M+. These results are summarized in Table 2.3.

3.1.3) Correlation of hemagglutinins with fimbriae

Three different classes of fimbriae were observed in *S. entomophila* by Transmission Electron Microscopy (TEM) and correlated with several observed haemagglutination patterns. MS-HA (type 1 fimbriae) was associated with nonchannelled fimbriae of 6 to 7 nm in width and 400-840 nm in length (Fig. 2.3 and 2.4 for comparison). This kind of fimbriae was observed to be arranged in clusters and in large numbers. These fimbriae were observed only in cultures grown at 30°C on LB plates. Only a small proportion of bacteria showed the presence of fimbriae (< 5 %).

Bacteria from the broth cultures with MR-HA activity, expressed channelled fimbriae of 4 to 5 nm wide (Fig. 2.5). About 10 % of cells showed this type of pili when grown on NB agar supplemented with milk. This percentage was increased to 50% after serial culture of bacteria under static conditions in broth supplemented with milk. However, TEM observation of this bacteria was difficult due to the presence of debris and particles attached to the cells. MR-HA associated fimbriae might have been expressed alone or with MS-HA fimbriae in the latter culture conditions.

A very fine nonchannelled kind of fimbriae of about 3 to 4 nm were associated with MR/K-HA (Fig. 2.6). These pili were poorly expressed on the surface of bacteria and were detected only in cultures grown in broth with no milk. A small proportion of cells showed these structures (< 10 %).

E. coli HB101 with MS-HA activity showed 70% of piliated cells in the culture. These type 1 fimbriae were morphologically different to those showed by *S. entomophila* strains (Fig. 2.7).

3.2) Haemagglutination pattern and fimbriae of *S. entomophila* nonpathogenic mutants

Nonpathogenic mutants were subjected to haemagglutination tests and TEM observation of fimbriae to analyze whether the loss of pathogenicity correlated with a change on haemagglutination and pili pattern. The mutants UC21 and UC7 were selected for this study. UC21 has a transposon (Tn*phoA*) inserted into its chromosome. The mutant was found to be nonpathogenic after the Tn*phoA* insertion and has apparently lost the ability of adhesion to the insect gut. Loss of pathogenicity observed in UC21 was associated with the Tn*phoA* insertion in the

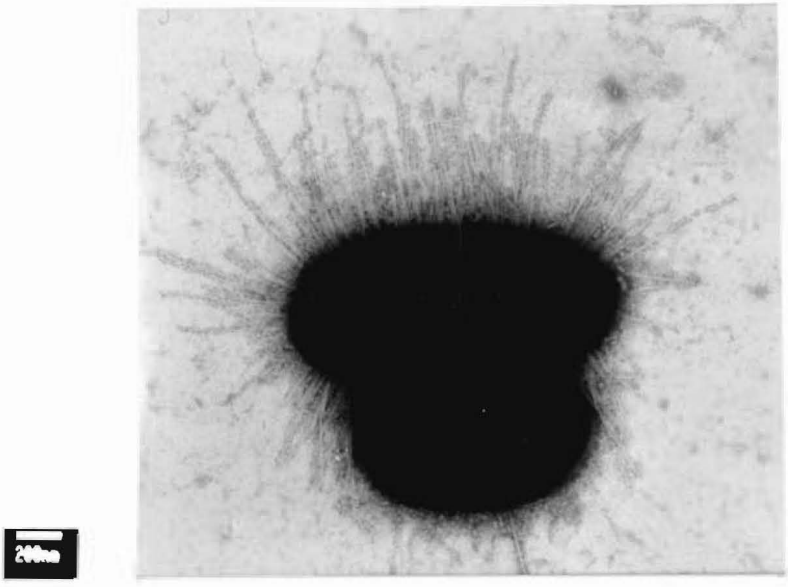


Fig. 2.3. Electron micrograph of negatively stained *S. entomophila* UC9 (Path$^+$) showing type 1 fimbriae. Cells were grown at 30°C overnight on LB plates and tested for haemagglutination activities. An aliquot of the culture was used for TEM observations. The cells show fimbriae type 1, which are associated with mannose sensitive haemagglutination (MS-HA). The proportion of piliated cells in this culture was less than 5%.

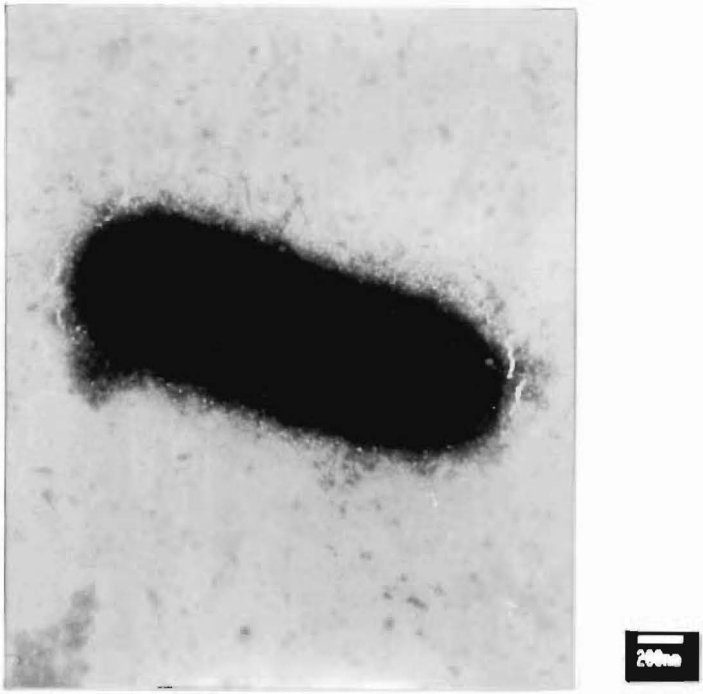


Fig. 2.4. Electron micrograph of negatively stained *S. entomophila* UC9 (Path$^+$) without fimbriae. Cells were grown at 30°C overnight. An aliquot of the culture was used for TEM observations. This cell is not surrounded by fimbriae. Only a small proportion of bacteria (< 5%) in the culture showed fimbriae in these conditions.

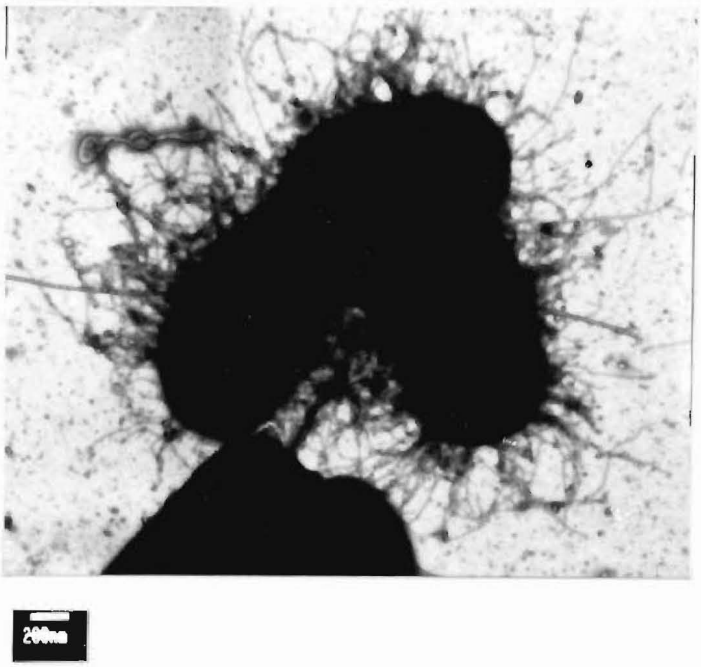


Fig. 2.5. Electron micrograph of *S. entomophila* A1MO2 (Path$^+$) showing MRE-HA fimbriae. Bacteria were cultured on NB agar supplemented with milk. These fimbriae were associated with mannose-resistant eluting haemagglutination (MRE-HA) and pathogenicity.

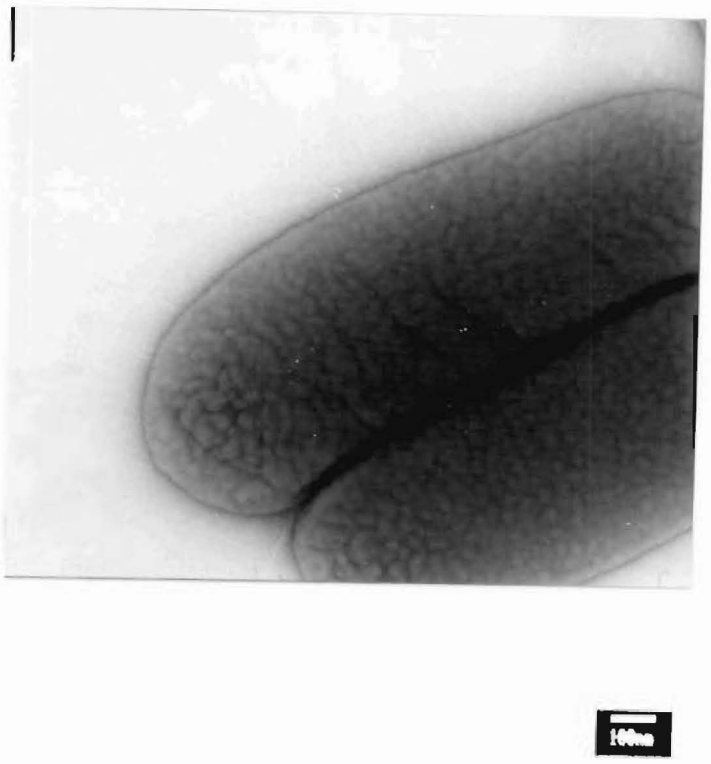
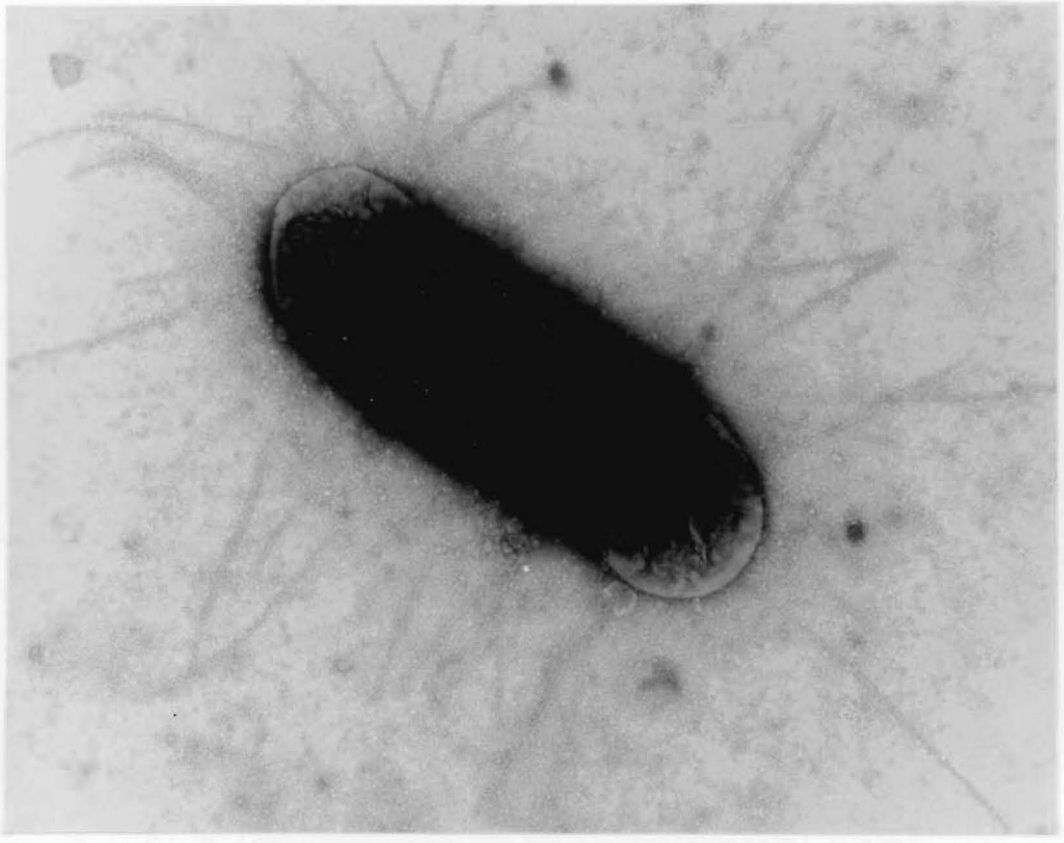


Fig. 2.6. Electron micrograph of *S. entomophila* UC9 (Path$^+$) showing MR/K-HA fimbriae. Bacteria were serially cultured at 30°C in NB and tested for haemagglutination. The cells agglutinated erythrocytes from sheep and ox previously treated with tannic acid. An aliquot was used for TEM observations. A small proportion of cells in the culture showed this type of pili.



200 nm

Fig. 2.7. Electron micrograph of negatively stained *E. coli* HB101 showing pili type 1. Bacteria were cultured at 30°C in NB supplemented with milk. The culture showed mannose-sensitive haemagglutination (MS-HA) activity. An aliquot of this culture was used for TEM examination. 70% of the cells in the sample expressed this type of pili.

amb1 locus and the suppression of pili production (Upadhyaya, *et al.*, 1992).

The strain UC7 is a Km^r spontaneous nonpathogenic mutant. Previous studies on this mutant (Wilson, 1988) suggested an alteration of its ability to adhere to the larvae gut. Therefore, alterations on the haemagglutination pattern and correlated fimbriae of these mutants would be expected to reveal interrelation with pathogenicity.

Growing conditions and haemagglutination tests were performed as described for A1MO2 and UC9. Haemagglutination tests showed MS-HA in both nonpathogenic isolates, similar to that observed for the pathogenic strains (Table 2.2). MR/K-HA was observed as well for both strains when they were grown in broth with no added milk in the first serial culture (Fig 2.8, row B3 and D3). When milk was present, haemagglutination of tanned sheep and ox erythrocytes (MR/K-HA associated) was not observed in UC21 (Table 2.3 and Fig. 2.2, row C3 and C7) but was present in UC7 (Table 2.3, Fig. 2.2, row D3 and D7). Agglutination of tanned sheep and ox erythrocytes was also noticed with A1MO2 and UC9 (Fig. 2.2, row A3,7 and B3, 7), yet it is related with MRE-HA, which seems to be evident for both, fresh and tanned blood cells. Unlike UC21 (Fig. 2.2, row C), UC7 (Fig 22, row D) was similar to the pathogenic strains in producing MRE-HA in broth supplemented with milk after 5 days of culture in the first series. The different concentrations of bacteria examined (3×10^8 to 3×10^{10} cells/ml), had no effect on the haemagglutination patterns. However, MRE-HA was observed in UC21 from older milk supplemented cultures (e. g. 4° serial culture, Fig. 2.8, row A) at similar cell concentrations. This result shows that UC21 is deficient in MRE-HA, compared with the $Path^+$ strains A1MO2 and UC9, yet it is not fully unable to express such activity. TEM observations of UC21 and UC7 showed a very low proportion of piliated cells (< 2 %) in all conditions tested. MS-HA associated pili were shown by both nonpathogenic strains UC21 (Fig. 2.9) and UC7 (Fig. 2.10). Similarly, MR/K-HA related pili were poorly presented in both cultures (results not shown). On the contrary, MRE/HA related pili were not found in any culture of either nonpathogenic strain, even though UC7 presented this kind of haemagglutination, resembling that of the pathogenic strains (Table 2.3). However, as it was previously mentioned, samples from milk supplemented broth were always very difficult to analyze by electron microscopy and fimbriae might have been masked.

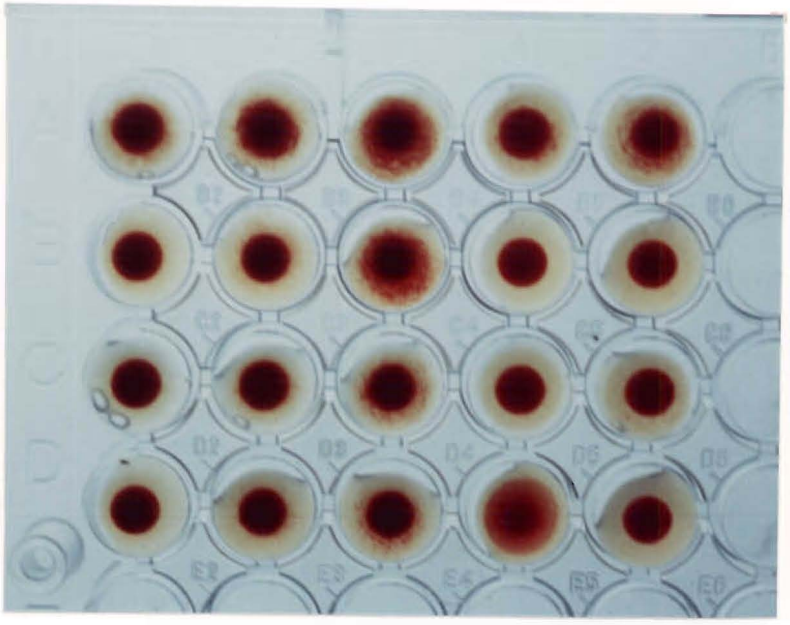


Fig. 2.8. Static settling test for mannose resistant eluting haemagglutination (MRE-HA) of *S. entomophila* nonpathogenic strains UC21 and UC7. Bacteria were cultured serially, in static conditions at 30°C in NB (NB) and in NB supplemented with milk (NBM). The test was performed after 5 days of growth in the 4th serial culture. Bacteria were mixed with erythrocytes in absence (M-) and presence (M+) of α-D-mannose and incubated at 4°C, 1 hr. Different strains in rows: A) UC21, NBM; B) UC21, NB; C) UC7, NBM; D) UC7, NB. Erythrocytes from: 1) sheep, M-; 2) sheep, M+; 3) sheep (tanned), M+; 4) guinea pig, M-; 5) guinea pig, M+.

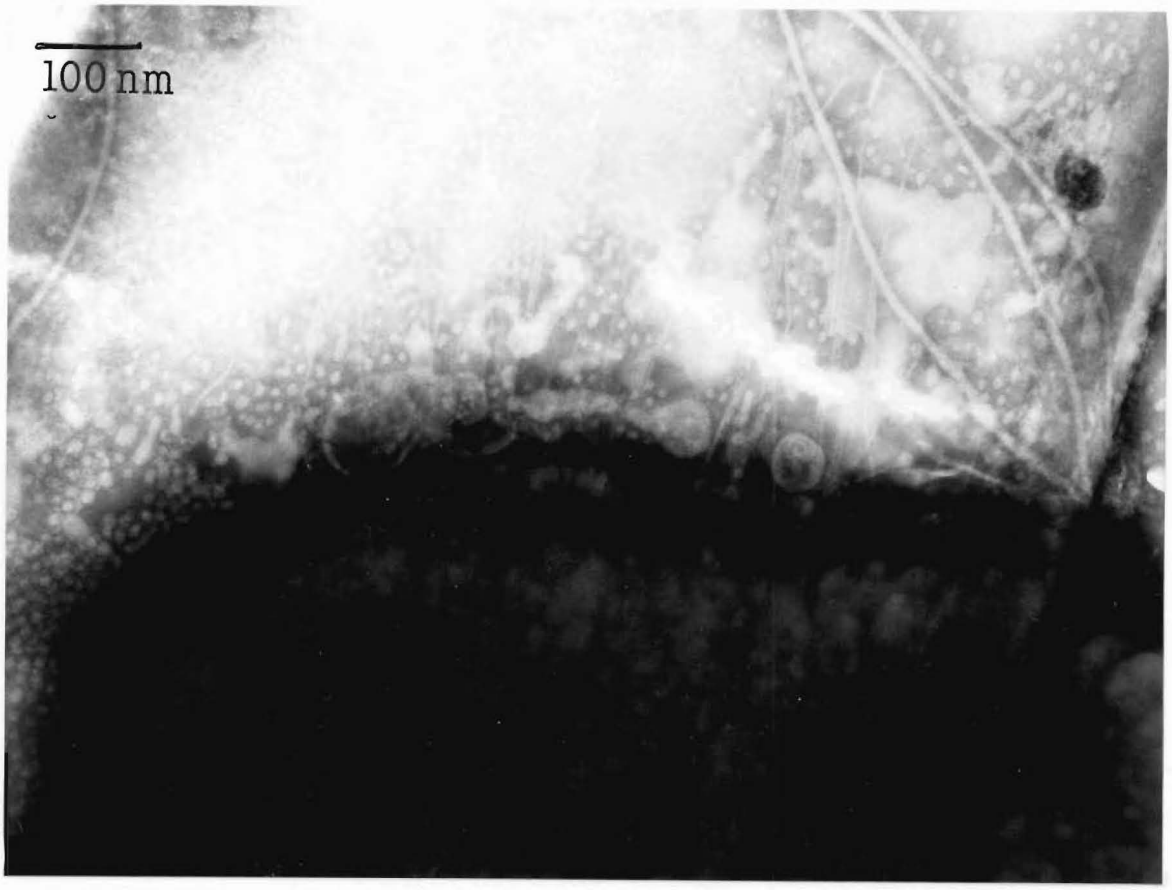


Fig. 2.9. Electron micrograph of negatively stained *S. entomophila* UC21 (Path⁻). Cells were serially cultured at 30°C and tested for haemagglutination activities. An aliquot of the culture was used for TEM observations. The cells show fimbriae type 1, which are associated with mannose sensitive haemagglutination (MS-HA). The proportion of piliated cells in this culture was < 2%.

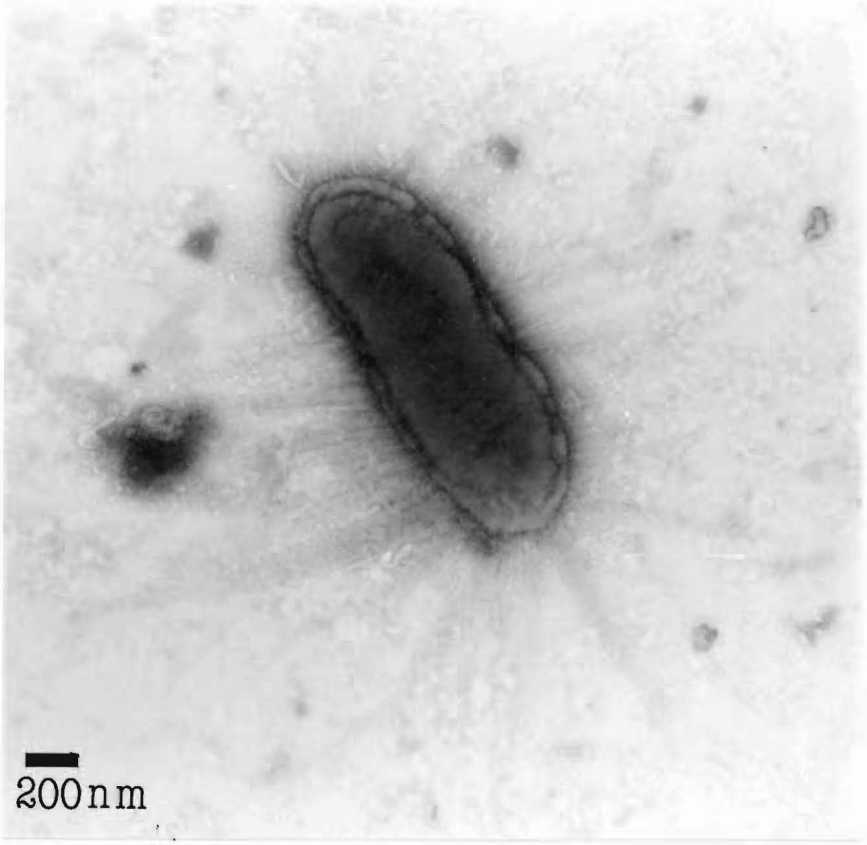


Fig. 2.10. Electron micrograph of negatively stained *S. entomophila* UC7 (Path⁻). Cells were serially cultured at 30°C and tested for haemagglutination activities. An aliquot of the culture was used for TEM observations. The cells show fimbriae type 1, which are associated with mannose sensitive haemagglutination (MS-HA). The proportion of piliated cells in this culture was < 2%.

4) DISCUSSION

Three different types of fimbriae were identified in the pathogenic *S. entomophila* strains A1MO2 and UC9 (Table 2.2). Several culture conditions for bacteria and erythrocytes from four different sources were used, since a complex array of hemagglutinins and fimbriae had previously been reported for *Serratia* spp. (Adegbola and Old, 1982). Even though *S. entomophila* produced three different classes of haemagglutination (MS-HA, MRE-HA, MRK-HA), the properties of each one could be separately identified in this study.

Type 1 fimbriae were demonstrated by strong agglutination of fresh horse erythrocytes and the observed inhibition of such effect by α-D-mannose (MS-HA). The fimbriae shown in Fig. 2.3 were observed only in cultures grown at 30°C on LB agar plates and therefore they were associated with MS-HA of horse erythrocytes. The affinity of this sort of pili to horse erythrocytes resembles the one found in *S. marcescens* (Parment, *et al.*, 1992), *S. marinorubra*, *S. liquefaciens* (Adegbola and Old, 1982) and *Salmonella typhimurium* (Korhonen, *et al.*, 1980) yet, it differs in the ability of some strains to agglutinate erythrocytes of guinea pig and sheep. Studies carried out with *S. marcescens* US5, isolated from a patient with urinary tract infection, showed that pili type 1 mediated adherence to uroepithelial cells (Yamamoto, *et al.*, 1985). However, a survey suggested that these pili are widely distributed among strains of *S. marcescens* (Kohno, *et al.*, 1984) and no clear evidence has so far been presented on the role of these pili in pathogenicity.

Type 3 fimbriae (Fig. 2.6) were associated with the ability of *S. entomophila* to agglutinate tanned ox and sheep erythrocytes in presence of α-D-mannose (MR/K-HA). Although type 3 fimbriae may appear in similar growing conditions to the type 1 fimbriae, the former present specific affinity to tanned erythrocytes and the latter, in *S. entomophila* agglutinate neither fresh nor tanned sheep and ox blood cells. The fimbriae shown in Fig. 2.6 were only observed in bacteria grown in NB with no milk supplement. These fimbriae were associated with MR/K-HA, since no other kind of fimbriae were observed in these culture conditions. Though MS-HA activity was also detected in the same cultures, MS-HA related fimbriae (Fig. 2.3) were not observed. MS-HA activity in these cultures was likely mediated by very small proportions of pilliated cells not detected in this study. The role of MR/K-HA fimbriae in pathogenic *Enterobacteriaceae*, if any, is unknown.

Another type of pili was found associated with mannose resistant haemagglutination (MR-HA) and was observed with fresh sheep, horse, guinea pig and ox erythrocytes (Table 2.3, Fig. 2.2 and 2.5). Because the effect was only stable at 4°C, it was defined as mannose resistant eluting-haemagglutination (MRE-HA). This sort of agglutination has not been reported for any *Serratia* species; it is a characteristic attribute of some MR-HAs of *E. coli* (Duguid, *et al.*, 1979). MRE-HA fimbriae have been involved in intestinal colonization by *E. coli* in humans and animals (Burrows, *et al.*, 1976; Evans and Evans, 1978; Evans, *et al.*, 1978; Jones and Rutter, 1972). On the other hand, the genes encoding the MR-HA fimbriae of *S. marcescens* US46, strain isolated from a patient with a urinary tract infection, have been cloned and sequenced (Mizunoe, *et al.*, 1991). It was shown, that these MR-HA genes were highly homologous to the *E. coli* Pap fimbriae which have been associated with acute infantile pyelonephritis and possess adhesins that bind to uroepithelial cells. MR-HA fimbriae have frequently been associated with pathogenicity (see Chapter 1).

Culture medium supplemented with milk was employed since *S. entomophila* produces an extracellular protease apparently induced by milk casein (Chapter IV). Some nonpathogenic mutants have been found deficient in protease production but no evidence of a direct role of the proteases on pathogenicity has been found (Chapter IV). Therefore, the regulation of synthesis of this enzyme might be associated with pathogenicity and/or the expression of virulence factors (e. g. pili). Coordinate regulation of virulence determinants has been reported in other pathogenic bacteria (see Chapter I), which supports this hypothesis . On the other hand, broth supplemented with aminoacids has led to optimal production of such determinants in *V. cholerae* and *E. coli* (Taylor, *et al.*, 1987; Evans, *et al.*, 1979). *S. entomophila* showed MRE-HA associated with fimbriae mainly produced in milk supplemented broth. This fact suggests a positive influence by some milk component(s) (e. g. milk casein and/or lactose) on the expression of these properties. A similar positive effect on pili expression by milk was observed for the reportedly non-fimbriated strain of *E. coli* HB101 (Fig. 2.7). Recent reports (Blomfield, *et al.*, 1991) showed that this strain actually contains the *fim* genes encoding fimbriae in *E. coli* K12 but it has lesions in a regulatory region involved in the control of fimbrial phase variation. The role of milk on expression and/or regulation of fimbriae in *S. entomophila* and *E. coli* is unknown. However, some network of regulatory elements influencing virulent determinants may be acting in these systems and be induced directly or indirectly in response to nutrient conditions. Synthesis of bacterial fimbriae in *Salmonella*

typhymurium was shown to be dependent on cyclic AMP and was subject to catabolite repression by a mechanism that depends on the metabolism of the repressing sugar (Saier, *et al.*, 1978). Expression of fimbriae in *E. coli* seems to be also controlled by the regulatory system CAP-cAMP (Göranson, *et al.*, 1989; Schmoll, *et al.*, 1990; Eisenstein and Dodd, 1982; Eisenstein, *et al.*, 1981), including the CFA/I fimbriae that mediates adhesion of enterotoxigenic strains to the mucosa of the small intestine in vertebrates (Karjalainen, *et al.*, 1991) and the Pap-pili-adhesin gene system, associated with acute infantil pyelonephritis (Forsman, *et al.*, 1992). Changes in levels of cyclic AMP probably due to the presence of lactose in *E. coli* and casein in *S. entomophila*, might induce fimbrial expression in these bacteria.

Results of this work have shown that the expression of hemagglutinins and/or fimbriae in *S. entomophila*, is promoted at 30°C and suppressed at 37°C. This feature has been observed in other *Serratia* strains (Adegbola and Old, 1982) and in the expression of virulence determinants including a pilus colonization factor in *Vibrio cholerae* (Taylor, *et al.*, 1987). The Pap fimbriae of *E. coli* is also subject to thermoregulation (Smyth and Smith, 1992). Therefore, temperature might be an important factor controlling expression of virulence genes in *S. entomophila.*

The nonpathogenic mutant UC21 was found to be deficient in the production of MRE/HA and/or related fimbriae. This fact supports previous results (Upadhyaya, *et al.*, 1992) on the role of *S. entomophila* pili as pathogenic determinants and suggests that those associated with MRE/HA are responsible for bacterial adhesion to the larvae gut and are associated with amber disease. Though UC21 was also shown defficient in protease production (Chapter III, Table 3.3) which might account for his Path$^-$ phenotype, it has been discussed by Upadhyaya, *et al.*, (1992) that the Tn*phoA* insertion in UC21 might be affecting an analogous of the pilus biosynthesis protein PilD of *Pseudomonas aeruginosa.* PilD is a pilin-specific leader peptidase that controls export of several enzymes including the exotoxin A, the alkaline phosphatase, the phospholipase C and the elastase of *P. aeruginosa.* The protease deficiency observed in UC21 supports the latter assumption. MRE/HA related pili were not observed by TEM in any culture of both nonpathogenic strains UC21 and UC7. However, contrary to UC21, UC7 did express MRE haemagglutination, similar to that of the pathogenic strains A1MO2 and UC9 (Table 2.3). Recent studies at the molecular level (Hacker, 1990; Minion, *et al.*, 1986; Uhlin, *et al.*, 1985; Mizunoe, *et al.*, 1991; Hultgren, *et al.*, 1989) have established the genetic distinction between the adhesin and the fimbrial structure. Thus, UC7 might be able

to express the MRE-HA fimbrial adhesin on the cell surface, even though the full pili is not expressed.

The role of hemagglutinins and related fimbriae in enterobacteria has been related with the establishment and colonization of pathogenic bacteria in humans and other mammals. The receptors on eukaryotic cells from different organisms such as mammals and insects may share common factors with affinity to bacterial fimbriae. In fact, hemagglutinins are widely distributed in commensal and free-living saprophytic bacteria (Adegbola and Old, 1982; Clegg and Gerlach, 1987) and their presence in virulent bacteria in hosts other than mammals should not be rare. The correlation of MRE/HA and associated fimbriae with pathogenicity of *S. entomophila* to insect larvae provided in this study is an example of the existence of general mechanisms of virulence in bacteria, where eukaryotic and prokaryotic organisms are linked by evolution in a common fashion.

Finally, the characterization of fimbriae in *S. entomophila* by haemagglutination tests reported here, provides a useful method to analyze *in vitro* the adhesion properties of a large number of strains, which is important for the study of virulence determinants in amber disease.

CHAPTER III

IDENTIFICATION, CLONING AND ROLE IN PATHOGENICITY OF THE GENES ENCODING THE EXTRACELLULAR PROTEASE OF *S. entomophila*

1) INTRODUCTION

A general feature of the genus *Serratia* is the production of a proteolytic activity. The synthesis of extracellular proteases has been demonstrated by Grimont, *et al.*, (1977). These extracellular proteases have been implicated in the pathogenicity of *S. marcescens* for insects (Flyg and Xanthopoulos, 1983; Kaska, 1976). Proteases from *S. marcescens* were shown to be toxic to larvae of the lepidopteran *Galleria mellonella*, causing insect death when applied by injection (Kaska, 1976). Proteases from other pathogenic bacteria have also tested toxic to insects, as it has been shown for proteases from *Pseudomonas aeruginosa* (Kucera and Lysenko, 1977) and *B. thuringiensis* (Lysenko, 1981). It has been demonstrated that protease-deficient mutants of the fish pathogen *Aeromonas hydrophila* have decreased virulence (Leung and Stevenson, 1988). The mutants did not produce lesions or kill rainbow trout when injected. It was suggested that proteases of *A. hydrophila* contribute to infection of fish by overcoming initial host defenses and by providing nutrients for cell proliferation. Protease-deficient mutants of the human pathogen *Vibrio cholerae* have shown also decreased virulence (Schneider and Parker, 1978). It has been suggested that these enzymes are involved in the disintegration of the protective layer of epithelial cells (Crowhter, *et al.*, 1987). The extracellular protease from *V. cholerae* has also been shown responsible for the processing of the cholera toxin to an active form (Booth, *et al.*, 1984).

Though proteolytic activity has been reported in *S. entomophila* (Grimont, *et al.*, 1988) the protease(s) have not been studied in details and its role in pathogenicity to larvae of *C. zealandica* has not been determined. This Chapter describes the identification, partial characterization and cloning of an extracellular protease gene of *S. entomophila.* In addition, it also describes the experiments to test its involvement in amber disease.

2) MATERIALS AND METHODS

2.1) Bacterial strains and vectors

The bacterial strains and plasmid vectors for cloning utilized in this work are described in Table 3.1, 3.2. For transformation and host for recombinant plasmids *E. coli* HB101 and DH5α were used when α-complementation for expression of ß-galactosidase was required. Bacterial strains were stored at -80°C in LB medium containing 25% (vol/vol) glycerol.

2.2) Media and growth conditions

Cells were regularly grown in LB broth or LB-agar. Antibiotics were added: 50 μg Ap, 50 μg Km, 30 μg Gm or 15 μg Tc per ml for selection with *E. coli* or some strains of *S. entomophila*. For some tests, 100 μg/ml of Km and 30 μg of Tc were used due to *S. entomophila* is highly resistant to low concentrations of antibiotics. *S. entomophila* strains were grown routinely at 30°C and *E. coli* at 37°C.

2.3) Milk and chitin overlay plates

Milk and chitin plates were used as indicators for protease and chitinase activity, prepared as described previously (Hines, *et al.*, 1988). Briefly, 10% (wt/vol) skim milk (commercial preparation) was dissolved in H_2O and autoclaved for 10 minutes. The milk solution was cooled at 37°C and 100 ml were added to 500 ml of autoclaved Luria-Bertani medium with 2% agar, prior to overlay onto plates. Antibiotics were added when necessary.

Chitin plates were prepared by overlaying a 20 ml agar base composed of nutrient agar (10 g/l) plus Bacto-agar (10 g/l) with a 10 ml layer of the same mixture supplemented with sterile hydrolysed chitin in a 2:3 ratio respectively. Hydrolysed chitin was prepared as reported (Shum, 1992) by treating 5 g of crystalline chitin (Sigma Chemical Co.) with 200 ml of 85% (wt/vol) ortho-phosphoric acid for 72 hours at 4°C. This mixture was washed and precipitated with distilled water three times. After the pH was adjusted to 7.4 with 2 M NaOH, the chitin was pelleted by centrifugation and then resuspended in phosphate-citrate buffer (75 mM NaH_2PO_4, 75 mM $Na_3C_6H_5O_7$) supplemented with 0.02% sodium azide as preservative.

2.4) Electrophoretic procedure (Gelatin-SDS-PAGE)

The method for SDS-PAGE containing gelatin as substrate for proteases was

Table 3.1. Bacterial strains used in this study

Strain	Relevant Characteristics	Reference
S. entomophila		
A1MO2	Wild-type; $Path^+$	T.A. Jackson
BC4B	Clonal selection from A1MO2; phage resistant; Ap^r $Path^+$	H.K. Mahanty
UC9	Clonal selection from A1MO2;Ap^r $Path^+$	H.K. Mahanty
UC7	Spontaneous Km^r mutant from A1MO2; Ap^r Km^r $Path^-$	H.K. Mahanty
UC21	Tn*phoA* mutant derivative from UC9; $Path^-$ Ap^r Km^r Gm^r	195
UC57	Tn*phoA* mutant derivative from BC4B; $Path^-$ Ap^r Km^r Gm^r	H.K. Mahanty
UC16	$Path^-$	T.A. Jackson
A20	Wild-type; $Path^-$	83
UC50	Tn*phoA* mutant derivative from UC9; $Path^-$ Ap^r Km^r Gm^r	This work
UC51	Tn*phoA* mutant derivative from UC9; $Path^-$ Ap^r Km^r Gm^r	This work
UC60	Tn*phoA* mutant derivative from UC9; $Path^-$ Ap^r Km^r Gm^r	This work
UC61	Tn*phoA* mutant derivative from UC9; $Path^-$ Ap^r Km^r Gm^r	This work
UC62	Tn*phoA* mutant derivative from UC9; $Path^-$ Ap^r Km^r Gm^r	This work
UC63	Tn*phoA* mutant derivative from UC9; $Path^-$ Ap^r Km^r Gm^r	This work
UC52	Tn*phoA* mutant derivative from BC4B; $Path^-$ Ap^r Km^r Gm^r	H. K. Mahanty
UC53	Tn*phoA* mutant derivative from BC4B; $Path^-$ Ap^r Km^r Gm^r	H. K. Mahanty
UC54	Tn*phoA* mutant derivative from BC4B; $Path^-$ Ap^r Km^r Gm^r	H. K. Mahanty
UC55	Tn*phoA* mutant derivative from BC4B; $Path^-$ Ap^r Km^r Gm^r	H. K. Mahanty
UC56	Tn*phoA* mutant derivative from BC4B; $Path^-$ Ap^r Km^r Gm^r	H. K. Mahanty
UC58	Tn*phoA* mutant derivative from BC4B; $Path^-$ Ap^r Km^r Gm^r	H. K. Mahanty
UC59	Tn*phoA* mutant derivative from BC4B; $Path^-$ Ap^r Km^r Gm^r	H. K. Mahanty
E. coli		
MM294	*endA hsdR pro supF44 endA1 thi*	127
SM10	*thi thr leu tonA lacY supE recA* [RP4.2 Tc::Mu] Ap^s Tc^s $Mu2^-$ Km^r Tra^+	168
HB101	F^- *pro leu thi lacY recA hsdR hsdM*	166
DH5α	F^- *lacZ* M15 *endA1 hsdR17 supE44 thi-1 gyrA relA1*	171

Table 3.2. Plasmids used in this study

Plasmid	Relevant Characteristics	Reference
pUC18	ColE1 *ori lacZ* Ap^r; cloning vector; 2.69 Kb	200
pLAFR3	pRK290 derivative with lambda *cos lacZ* and polycloning sites of pUC9; RP4 (IncP-1) *ori* Tc^r; cloning vector; 22 Kb	184
	BHR IncP-1 pRK290 with Tn*phoA*:PhoA- Tc^r Km^r	190
pRT291	Inc-1 *tra* Spc^r Gm^r Sm^r	10
pPH1J1	ColE1::pKK2 IncP-1 *tra* Km^r	51
pRK2013	Km^r *lac*$^+$	
pPRO1	pLAFR3 cosmid clone from *S. entomophila* UC9 library with a ~26 Kb DNA insert containing the protease gene(s); Tc^r.	This work
pPRO2	*Bam*HI insert fragment of ~4 Kb containing *S. entomophila* protease gene(s) from pPRO1 cloned into pUC18; Ap^r	This work
pPRO3	*Bam*HI insert fragment of ~4 Kb containing *S. entomophila* protease gene(s) from pPRO1 cloned into pUC18; Ap^r	This work
pPRO4	*Bam*HI insert fragment of ~8.5 Kb containing *S. entomophila* protease gene(s) from pPRO1 cloned into pUC18; Ap^r	This work
pPRO5	*Bam*HI insert fragment of ~11.5 Kb containing *S. entomophila* protease gene(s) from pPRO1 cloned into pUC18; Ap^r	This work
pPRO6	*Bam*HI insert fragment of ~9.1 Kb containing *S. entomophila* protease gene(s) from pPRO1 cloned into pUC18; Ap^r	This work
pPRO7	*Bam*HI insert fragment of ~13.5 Kb containing *S. entomophila* protease gene(s) from pPRO1 cloned into pUC18; Ap^r	This work
pPRO8	*Bam*HI insert fragment of ~6.3 Kb containing *S. entomophila* protease gene(s) from pPRO1 cloned into pUC18; Ap^r	This work
pPRO9	*Bam*HI insert fragment of ~10.3 Kb containing *S. entomophila* protease gene(s) from pPRO1 cloned into pUC18; Ap^r	This work

modified from the system reported by Heussen and Dowdle (1980). The resolving mini-gel slabs (60 x 70 x 1 mm) were cast from a mixture prepared from stock solutions as follows:

H_2O	1.3 ml
Acrylamide (29 g%) and bisacrylamide (1 g%)	1.7 ml
1.5 M Tris-HCl, pH 8.8	1.3 ml
1% Gelatin (in H_2O)	0.5 ml
10% SDS	0.15 ml
10% Ammonium persulfate	0.05 ml
TEMED	2 µl
Total volume	5 ml

The resolving gels were overlaid with 0.3 M Tris-HCl (pH 8.8) and 0.05% gelatin, prewarmed at 65 °C. After 30 min at room temperature, the stacking gel was cast from the following mixture:

H_2O	1130 µl
Acrylamide (29 g%) and bisacrylamide (1 g%)	330 µl
0.5 M Tris-HCl, pH 6.8	500 µl
10% SDS	20 µl
10% Ammonium persulfate	20 µl
TEMED	2 µl
Total volume	2 ml

Polymerization was allowed 1 hr at room temperature. Tank buffer (0.025 M Tris-HCl pH 8.3, 0.192 M glycine, 0.1% SDS) was added to the corresponding reservoirs and the gel was pre-run for 30 min (100 V). Samples were mixed with 15 μl of loading buffer (0.125 M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 10% 2-ß-mercaptoethanol) and added to the wells in a final volume of 30 μl. The samples containing MW markers or bacteria were boiled for 2 min. Electrophoresis was performed on a BioRad minigel apparatus at 4 °C, 217 V for 50 min. The gel was washed 1 hr in Triton X-100 at room temperature to remove SDS and restore enzyme activity. Then, it was incubated overnight at 37 °C in 0.1 M Tris-HCl pH 8. The gel was stained in Coomassie blue solution (0.125% Coomassie Blue R-250, 50% methanol, 10% acetic acid) during 2 hr and immersed for 48 hrs in destaining solution (7% acetic acid, 5% methanol).

2.5) Tn*phoA*-induced mutagenesis

Tn*phoA* mutagenesis of *S. entomophila* UC9 was carried out as previously reported by Upadhyaya, *et al.*, (1992). Plasmid pRT291 containing Tn*phoA* was transferred from the donor *E. coli* SM10 to *S. entomophila* UC9 by conjugation. Five hundred μl of each overnight broth culture were mixed in a microcentrifuge tube. The mixture was centrifuged 2 min at 6K, resuspended in 1 ml of LB broth and incubated for 5 hrs at 30°C. Transconjugants were selected by resistance to suitable antibiotics and tested for pathogenicity. In a second mating, the plasmid pPH1JI was mobilized from *E. coli* MM294 to the transconjugant UC9 carrying pRT291. One ml of each overnight broth culture was centrifuged and resuspended in 100 μl of LB. The bacteria were mixed together and spread onto agar plates containing antibiotics. The double transconjugants (UC9 bearing pRT291 and pPH1JI) were selected for pPH1JI (Gm^r) and retention of Tn*phoA* (Km^r). A second selection for loss of Tc^r confirmed the occurrence of transposition. Insertion of the transposon into UC9 genome, has been previously confirmed by Southern blot analysis of Tn*phoA*-induced mutants (Upadhyaya, *et al.*, 1992). The frequency of conjugation in this experiment was 2×10^{-8} per recipient UC9 cell and 95% of transconjugants were Tc^s.

2.6) DNA isolation, restriction mapping and cloning

Restriction enzyme digestion was performed as suggested by the manufacturers, New England Biolabs and Gibco Bethesda Research Laboratories or Life Technologies Ltd. Agarose gels electrophoresis for DNA samples was carried

out as described (Maniatis, *et al.*, 1982) The construction of recombinant plasmids, the isolation of plasmid DNA and transformation of *E. coli* were performed by standard methods (Sambrook, *et al.*, 1989). Restriction endonuclease fragments for cloning, were purified from low melting point agarose gel electrophoresis (Bethesda Research Laboratories).

2.7) Electroporation

Cells were grown in 100 ml of LB with suitable antibiotics to OD_{600} of 0.5-0.8. The cells were placed on ice for 30 min and centrifuged 15 min, 6 K, 4°C (Sorvall GSA rotor). The pellet was washed and centrifuged twice with 100 ml of cold H_2O. Finally, the cells were resuspended in 300 µl of 10% glycerol (~3 X 10^{10} cells/ml), frozen in aliquots of 40 µl and stored at -80°C. Electroporation, also called electro-transformation, was performed on a Gene Pulser apparatus (BIO-RAD). Cells were thawed at RT and placed on ice. Forty µl of the cell suspension were mixed with 20-400 ng of DNA resuspended in 1-2 µl of H_2O. The conditions on the Gene Pulser apparatus were set at 25 µF, 2.5 KV and 200 Ω for 2mm cuvettes. One pulse was applied before the addition of 1 ml of SOC medium (2% triptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl, 10 mM $MgSO_4$, 20 mM glucose). Cells were incubated at 30 or 37°C for 1 hr and plated out on selective media.

2.8) Bioassays with insect larvae

Procedures of the bioassays for pathogenicity tests are described in Chapter IV, section 2.6.

3) RESULTS

3.1) Identification of *S. entomophila* extracellular protease

S. entomophila was previously shown to be able to hydrolyse gelatin (Grimont, *et al.*, 1988). Proteolytic activity in the present work was first demonstrated by growing the bacteria on agar-plates containing skim milk as previously reported (Hines, *et al.*, 1988). Milk clearing zones were produced by the pathogenic strains A1MO2 and UC9 (Table 3.3). These clearing zones were related with hydrolysis of milk casein, thus demonstrating the proteolytic activity. The size of the halos surrounding the colonies were determined as a semiquantitative measure

Table 3.3. Protease and chitinase activity of pathogenic and nonpathogenic *S. entomophila* strains

		Protease activity[a]		Chitinase activity[b]
Strain	**Path**	**24 hrs**	**48 hrs**	**48 hrs**
A1MO2	+	3.5	6	6.5
UC9	+	3.5	6	5.5
BC4B	+	4.5	10	5.5
A20	-	4.5	10	5.5
UC7	-	2.1 *	2.1	3.5 *
UC21	-	2.5 *	2.5	5.5
UC50	-	3.5	5	5.5
UC51	-	1.5 *	2.6	5.5
UC52	-	5.5	11	5.5
UC53	-	5.5	11	5.5
UC54	-	4.5	9	5.5
UC55	-	5.5	11	5.5
UC56	-	4.5	10	5.5
UC57	-	1.5 *	2.6	3.5 *
UC57/pPRO1	-	3.0	4.8	ND[c]
UC58	-	4.5	11	5.5
UC59	-	4.5	11	5.5
UC60	-	1.5 *	2.6	5.5
UC61	-	1.5 *	1.5	3.5 *
UC62	-	1.5 *	1.5	5.5
UC63	-	3.5	5.5	5.5

[a] Colonies were grown on LB medium supplemented with milk and appropriate antibiotics at 30°C. Halo diameters in mm are reported representing the mean of 5 colonies per strain. Variability of the halo diameters was no more than 10%. The colonies themselves had a diameter of ca. 1.5 mm after 24 hrs and 2-2.5 mm after 48 hrs incubation.

[b] Colonies were grown on LB medium supplemented with chitin at 30°C. Halo diameter in mm are reported representing the mean of 5 colonies per strain. Variability of the halo diameters was no more than 10%. The colonies had a diameter of ca. 1.5 mm.

[c] ND= Not determined.

* Denotes deficiencies either in protease or chitinase activity.

of the degree of protease activity.

Proteases of *S. entomophila* were analyzed by electrophoresis in polyacrylamide gels containing the detergent SDS (SDS-PAGE) and gelatin as described for similar systems (Heussen and Dowdle, 1980). Gelatin works as *in situ* substrate for proteases and clear zones corresponding to protein bands appeared after the gel was stained with coomasie blue (Fig. 3.1). Only one protease band was detected in culture supernatants from UC9 grown in the presence and absence of milk supplement (lanes 2,6 and 4,8 respectively). The proteolytic activity showed by these protein bands from cells growing with milk was apparently stronger than those without milk. The same band was observed when lysed bacteria were analyzed, although it was very faint (lane 5). No other proteases were detected in the bacteria by this method. Therefore, *S. entomophila* seems to produce only one protease and this enzyme is secreted to the external medium. The molecular weight of this protease was estimated as approximately 49 kD, by comparison with molecular weight markers in a range of 14.4K to 97.4K (SDS-PAGE not shown).

3.2) Cloning of DNA encoding protease activity

The *S. entomophila* UC9 cosmid library constructed in *E. coli* HB101 and pLAFR3 as the vector (Upadhyaya, *et al.*, 1992), was screened for extracellular protease activity on agar-milk plates. From 3,000 colonies screened, 29 colonies displaying different halo size were isolated. One of these colonies showing a clear halo of 5 mm diameter was further analyzed. The isolated cosmid, named pPRO1, was double digested with *Eco*RI and *Hind*III to isolate the insert from the recombinant cosmid. The DNA insert from the *S. entomophila* genome was approximately 26 Kb, calculated from the restriction pattern in agarose gels. The genes encoding the protease activity were contained in this fragment. Therefore, this DNA was further subcloned to determine the region containing the structural genes for protease.

Plasmid pPRO1 was partially digested with *Sau*3A and analyzed by agarose gel electrophoresis. DNA showing partial restriction in the gel was ligated with the vector pUC18 previously digested with *Bam*HI and treated with CIP to avoid religation. *E. coli* DH5α were transformed with the ligation mixture and selected on milk-agar plates in the presence of Ap. Nine colonies showing protease activity were further analyzed. Plasmids were isolated and double digested with *Eco*RI and *Hind*III

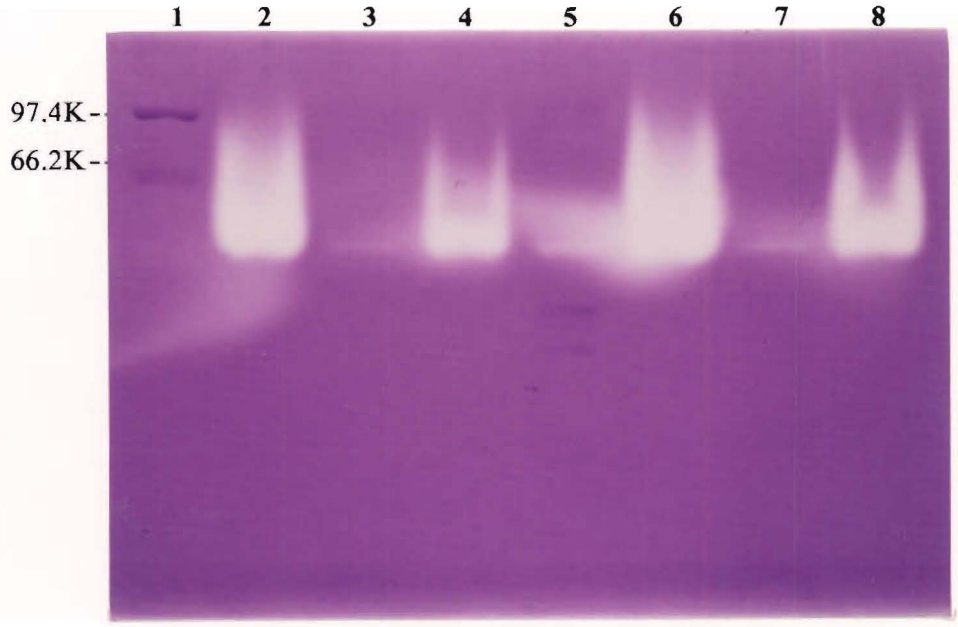


Fig. 3.1. Gelatin-SDS-polyacrylamide gel stained with coomassie brilliant blue showing *S. entomophila* extracellular proteases. Cells were grown at 30°C overnight in LB broth with (m+) and without (m-) milk. Antibiotics were added when needed. The cultures were centrifuged to pellet the cells. Culture supernatants (sp) were removed and filter sterilized. Cells (lc) were lysed by boiling. Culture supernatants were not boiled. Sp and lc were used for electrophoresis. Line: 1) Molecular weight markers; 2) *S. entomophila* UC9, m+, sp (0.2 μl); 3) *E. coli* DH5α/pPRO2, m+, sp (6 μl); 4) UC9, m-, sp (0.2 μl); 5) UC9, m+, lc (0.2 μl); 6) UC9, m+, sp (0.4 μl); 7) *E. coli* DH5α/pPRO2, m+, sp (12 μl); 8 UC9, m-, sp, (0.4 μl). Bands in lane 5 are proteins of the lysed cells UC9. *E. coli* DH5α was previously shown unable to produce protease activity on milk plates.

(Fig. 3.3). The restriction pattern showed different insert sizes ranging from 4 to 13.5 Kb. All these positive clones were found to share a common DNA fragment of 1.4 Kb, which presumably contained the structural genes for protease.

One plasmid with a 4 Kb DNA insert containing the protease genes was named pPRO2 (Fig. 3.3, lane d). Culture supernatants from *E. coli* DH5α carrying this plasmid and grown in milk supplemented broth, was analyzed by gelatin SDS-PAGE. Parallel analysis of culture supernatants from *S. entomophila* demonstrated similar protease bands in both lanes of the gel (Fig. 3.1). This results demonstrates that the 4 Kb insert in pPRO2 contains the structural genes encoding the extracellular protease of *S. entomophila.*

Protease activity produced by *E. coli* bearing either pPRO1 or its derivatives including pPRO2, was apparently lower than the activity level produced by wild type *S. entomophila,* as observed by the size of the halo on agar-milk plates. Similar differences were observed in gelatine SDS-PAGE (Fig. 3.1), since 30 fold dilution sample of *S. entomophila* supernatants (lane 2) produced more protease activity than its undiluted counterpart produced by the protease genes in *E. coli* (lane 3).

3.3) Generation and partial characterization of *S. entomophila* protease activity deficient mutants

In order to understand the pathogenicity of *S. entomophila,* mutants were generated by transposon mutagenesis as described in Materials and Methods (section 2.5). Two hundred Tn*phoA*-induced mutants were tested for protease and chitinase activities. The same mutants were also tested for loss of pathogenicity to *C. zealandica* larvae by bioassays. Results are summarized in Tables 3.3, 3.4 and Fig. 3.4. Briefly, 40 mutants showed reduced zones of proteolysis (Prot-) on milk agar plates (Fig. 3.2). One mutant showed decreased chitinase activity (Chi-) and six mutants were found to be non-pathogenic (Path$^-$; Table 3.3). Therefore, 36 out of 40 protease deficient mutants remained virulent (data not shown). Loss of pathogenicity in the mutants named UC51, UC60, UC61 and UC62 (Fig. 3.4), correlated with deficiencies in protease activity (Table 3.3 and 3.4). The mutant UC61 (Path$^-$, Prot-) was also deficient in chitinase activity (Table 3.3). Two other mutants named UC50 and UC63, were found to be non-pathogenic (Path$^-$), yet they retained the protease activity of the wild-type parent strain (Table 3.3). No apparent growth defects were observed in these mutants.

Table 3.4. Bioassay of nonpathogenic isolates after Tn*phoA* mutagenesis[a]

Strain	AFE[b]	NET AFE[c]	AC[d]
A1MO2	70	60	80
UC16	10	0	0
UC50	0	NA[e]	10
UC51	20	10	10
UC60	40	30	30
UC61	10	0	30
UC62	60	50	30
UC63	100	90	33

[a] Transposon mutagenesis was carried out in the Path$^+$ strain UC9. Results of the selected nonpathogenic mutants are presented. The positive control was the path+ strain A1MO2. The Path$^-$ strain UC16 was used as a negative control. Results based on 10 larvae per treatment after 6 days of inoculation.

[b] Anti-feeding effect (AFE) and [d]amber coloration (AC) are in percentage of larvae showing these amber disease symptoms.

[c] NET AFE represents the arithmetic difference between the AFE of each strain and the AFE of the negative control (e.g. [AFE of A1MO2] - [AFE of UC16] = 60).

[e] Not applicable

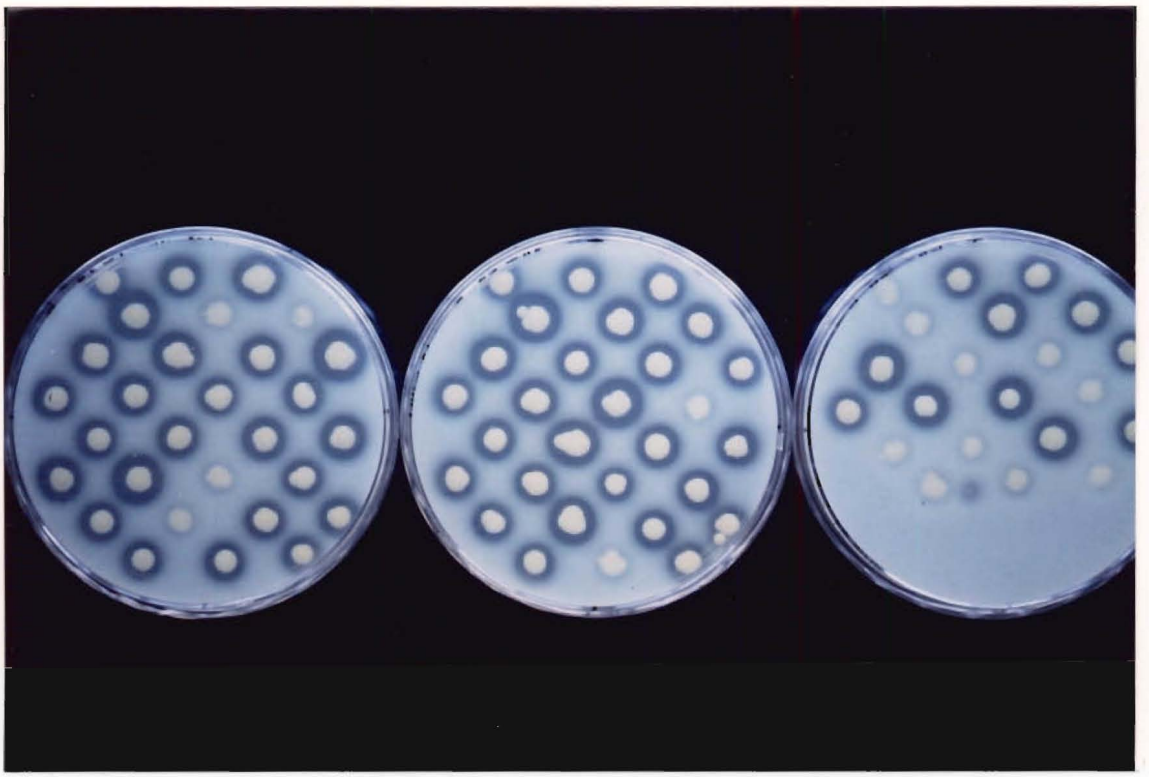


Fig. 3.2. Skim milk plate assay for selection of protease-deficient *S. entomophila* mutants. Transposon mutagenesis of the Path$^+$ strain UC9 was carried out. Mutants were grown over night at 30°C on LB supplemented with milk and appropriate antibiotics.

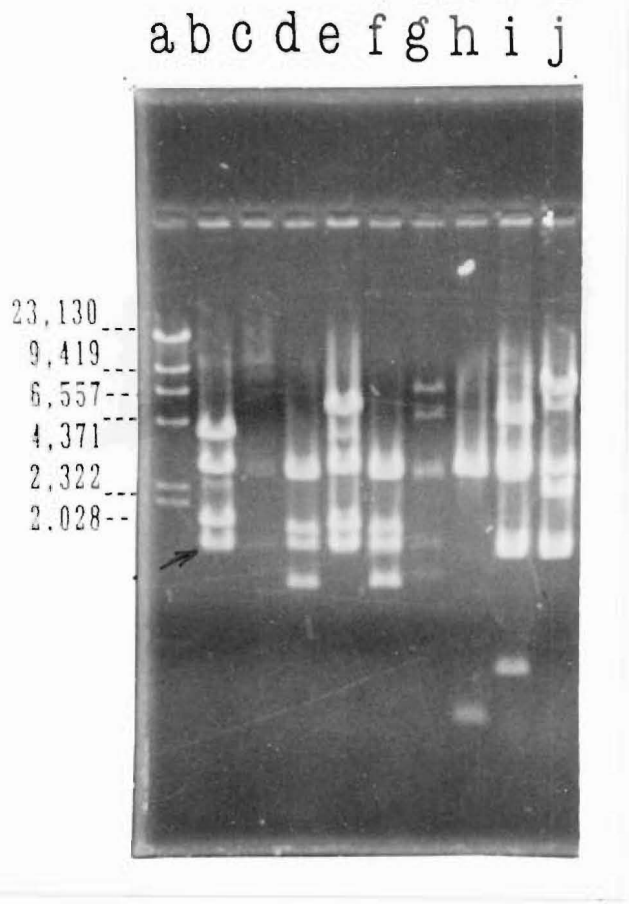


Fig. 3.3. Electrophoresis in agarose gel of recombinant plasmids carrying the *S. entomophila* protease gene(s). Plasmid pPRO1 containing protease gene(s) was partially digested with *Sau*3A and ligated with pUC18 previously digested with *Bam*HI. *E. coli* DH5α were transformed with the ligation mixture and colonies showing protease activity were selected on agar-milk plates. The restriction pattern of the recombinant plasmids digested with *Hind*III and *Eco*RI shows insert sizes ranging from 4 to 13.5 Kb. Clones expressing protease activity share a common DNA fragment of 1.4 Kb (lanes b, c, d, e, f, g, i, j), which is not present in a negative clone (lane h). The arrow in the picture indicates the 1.4 Kb fragment. Lanes in the 0.7% agarose gel: a) Lambda/*Hind*III; b) pPRO4; c) pPRO5; d) pPRO2; e) pPRO6; f) pPRO3; g) pPRO7; h) Negative clone; i) pPRO8; j) pPRO9.

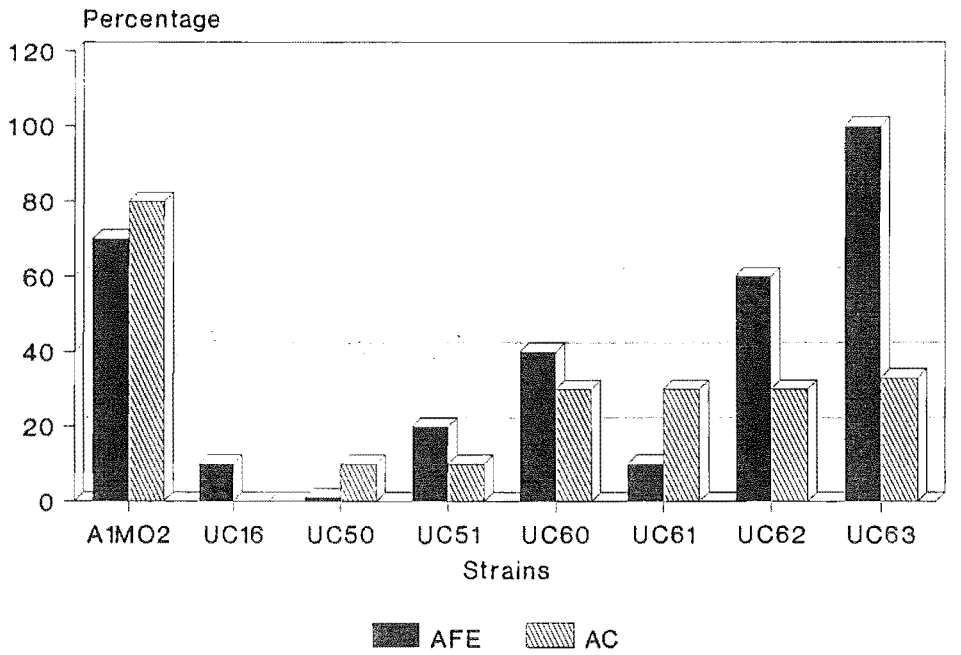


Fig. 3.4. Insect larvae bioassay of *S. entomophila* Tn*phoA* mutants. Transposon mutagenesis was carried out in the Path$^+$ strain UC9. Results of the selected nonpathogenic mutants are presented. The positive control was the Path$^+$ strain A1MO2. The Path$^-$ strain UC16 was used as negative control. Bars represent percentages of larvae showing anti-feeding effect (AFE) and amber coloration (AC). Results[1] based on 10 larvae per treatment after 6 days of inoculation.

[1] Bioassay performed by AgResearch, Lincoln.

Other pathogenic (Path$^+$) and non-pathogenic *S. entomophila* strains, provided by H.K. Mahanty, were also screened for protease and chitinase activities. As it is shown in Table 3.3, the Path$^+$ strain, BC4B, produced stronger protease activity than that showed by the wild type strain A1MO2. The naturally occurring Path$^-$ strain A20, produced a halo of similar size as the one produced by BC4B. In a similar way, the Path$^-$ Tn*phoA* mutants UC52, UC53, UC54, UC55, UC56, UC58 and UC59 exhibited protease activity. Conversely, the non-pathogenic Km^r spontaneous mutant UC7 displayed reduced protease activity. Similar feature was observed in the Path$^-$ Tn*phoA* mutants UC21, and UC57. On the other hand, only one Path$^-$ strain from this group (UC7), showed reduction in chitinase activity.

No mutants were found completely lacking protease activity. A small clear halo was observed around bacteria after several days of growth on agar-milk plates in all protease deficient mutants tested so far.

3.4) Complementation assay of the protease deficient mutant UC57 with the protease gene(s)

The cloned *S. entomophila* protease genes were tested for complementation of the nonpathogenic mutant UC57 in order to determine the role of this extracellular enzyme in amber disease. Since reduced levels of protease activity observed in the Tn*phoA* mutant UC57 correlated with loss of virulence, it was hypothesized that the protease genes might restore pathogenicity.

Plasmid pPRO1 (Tc^r) was introduced into UC57 (Ap^r, Km^r) by electroporation. Plasmid pLAFR3 was also introduced to be used as control. Bacteria carrying the plasmids were selected on agar-milk plates containing Ap, Km and Tc. Colonies displaying protease activity in these conditions and bearing pPRO1 were tested for Gm^s; this phenotype indicates loss of pPH1JI (Gm^r) due to incompatibility with the vector pLAFR3. Plasmid isolation from the selected colonies and further transformation of *E. coli* HB101 with this DNA was performed to confirm the presence and stability of the plasmids in UC57. No changes were detected in the restriction pattern of plasmids contained in transformed HB101.

UC57 carrying pPRO1 showed protease activity almost resembling that observed in pathogenic strains UC9 and A1MO2 (Table 3.3). These bacteria were used for bioassays with larvae of *C. zealandica* to test for pathogenicity restoration.

Parallel assays were performed with bacteria carrying plasmid vector and with the pathogenic strain UC9.

Larvae were fed with these bacteria as described in Materials and Methods (Chapter IV). Observations were made on feeding behaviour and development of amber coloration. Results are summarized in Fig. 3.5 and in Table 3.5. As was expected, most larvae fed with UC9 (94.4%) presented characteristic symptoms of amber disease: anti-feeding effect (AFE) and amber coloration (AC). Conversely, such symptoms were not observed in larvae fed with the Path$^-$ UC57 carrying plasmid vector alone. Amber disease symptoms were not observed either in the larvae fed with UC57 bearing pPRO1. Since pathogenicity was not restored in UC57 expressing protease genes, it was suggested that the *S. entomophila* extracellular protease does not have a direct role in amber disease.

4) DISCUSSION

Proteolytic activity was detected in *S. entomophila* and an apparent single extracellular protease was identified by gelatin SDS-PAGE (Fig. 3.1). Single exoproteases have also been reported in *Serratia* spp. (Miyata, *et al.*, 1970; Rydén and Hofsten, 1968) which is in agreement with this result. However, Grimont *et al.*, (1977) found a high degree of protease heterogeneity in several *Serratia* species. Diversity of proteases in *S. entomophila* is not fully excluded by this result, since a single band observed in one-dimensional gel might contain several proteases. However, these proteins would be very similar in size as judged by the same migration pattern in SDS-PAGE, though with different specificities and or kinetics. It has been shown that the several proteases observed in *S. marcescens* are the result of different forms of enzyme (Schmitz and Braun, 1985). It is not clear whether this might be caused by processing reaction (modification) or the existence of several genes encoding isoenzymes. The inability to isolate mutants totally lacking protease activity in this work supports the latter assumption.

Parallel protease bands detected in lysed UC9 cells (Fig. 3.1, lane 5) and in culture supernatants (lane 2, 4), may reflect in the first case the *de novo* synthesized protease still in the cells before secretion, or the existence of membrane-bound enzyme. This location of some proteases has been reported in *Serratia* spp. (Rydén and Hofsten, 1968).

Proteolytic activity and hence synthesis of proteases was observed in absence

Table 3.5. Bioassay of *S. entomophila* Path⁻ mutant UC57 carrying protease gene(s)

Strain	AFE[a]	NET AFE[b]	AC[c]
UC9	94.4	83.3	94.4
UC57/pLAFR3	11.1	0	11.1
UC57/pPRO1	14.3	3.2	21.4

[a] Anti-feeding effect (AFE) and [c]amber coloration (AC) are in percentage of larvae showing these amber disease symptoms.

[b] NET AFE represents the arithmetic difference between the AFE of each strain and the AFE of the negative control (UC57/pLAFR3) (e. g.[94.4]-[11.1]= 83.3).

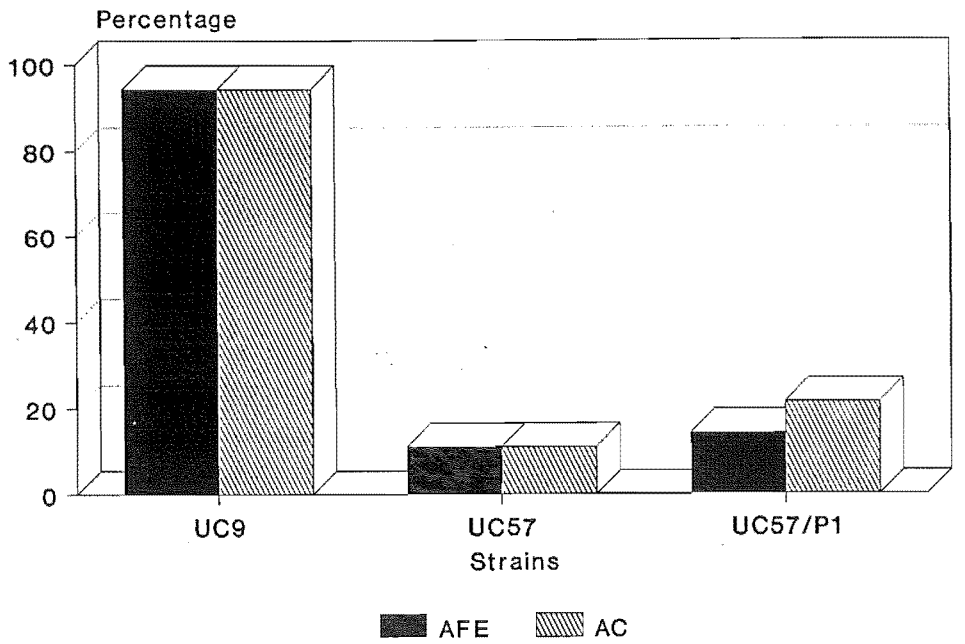


Fig. 3.5. Insect larvae bioassay of *S. entomophila* Path⁻ mutant UC57 carrying protease gene(s). Plasmid pPRO1 containing the extracellular protease gene(s) of *S. entomophila* was introduced in UC57 to test for complementation. The positive control was the Path$^+$ strain UC9 and the negative one was UC57 carrying the vector pLAFR3. Anti-feeding effect (AFE) and amber coloration (AC) are represented in percentage of larvae showing these amber disease symptoms. Third instar larvae were used for the bioassay. Data of 10 days after treatment are presented.

of milk supplement, synthesis that seems to be constitutive. Some increase of activity level was noticed when bacteria were grown in the presence of milk (Fig. 3.1, lanes 2 and 6). Induction of protease synthesis by milk casein is suggested. Bromke and Hammel (1978), reported gelatin as an inducer of *S. marcescens* proteases.

The structural gene for the extracellular protease of *S. entomophila* was cloned in a 4 Kb DNA fragment. This protease gene was expressed in *E. coli*, though to a lesser degree than in *S. entomophila*, as judged by the halo size (data not shown) and protein band observed in gelatin SDS-PAGE (Fig. 3.1, lanes 3 and 7). Reduced expression of a cloned *Serratia* sp. protease gene(s) in *E. coli* was also observed by Nakahama, *et al.*, (1986). Some DNA regions or cistrons[2] required for efficient transcription and/or secretion may be missing from the cloned genes (pPRO1 derivatives) causing low levels of protease activity. However, it is evident that transcription and/or translation signals of the *S. entomophila* protease gene(s) are recognized by *E. coli*.

The molecular weight of the cloned protease was estimated to be about 49 kD and is consistent with other *Serratia* spp. proteases (Nakahama, *et al.*, 1986).

S. entomophila mutants were generated by transposon mutagenesis. A correlation was observed in some isolates between loss of pathogenicity and deficiencies in protease activity. Therefore, it is suggested that this extracellular protease might be involved in the development of amber disease. Even though some mutants were also found expressing reduced levels of protease activity and remained virulent, no mutants were detected that were totally lacking protease activity. Small amounts of this protein might be enough for executing their function in pathogenicity.

On the other hand, the naturally occurring nonpathogenic strain A20 produced higher levels of protease activity than the Path$^+$ strains A1MO2 and UC9, and similar level to those showed by BC4B. This fact suggests that the role of proteases in amber disease would be secondary, since the sole presence of proteases does not cause pathogenicity.

Proteases from several bacteria have shown toxicity to insects and have been

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[2] Cistron represents the genetic unit equivalent to a gene in comprising a unit of DNA encoding a protein (Lewin, 1987).

also associated with virulence. The toxic activity seems to be related with a selective destruction of haemocytes (Lysenko, 1981) and cecropine (Flyg and Xanthopoulos, 1983) in the larval hemolymph, affecting consequently the defense mechanisms of the insect. This effect has been observed in the lepidopteran *Galleria mellonella* with proteases of *S. marcescens* (Kaska, 1976), *P. aeruginosa* (Kucera and Lysenko, 1977) and *B.thuringiensis* (Lysenko, 1981). Proteases from *P. aeruginosa* have also been implicated in some human infections (Holder and Haidaris, 1979). The protease activity of *S. marcescens* was also related with pathogenicity in experimental pneumonia in guinea pigs and mice (Lyerly and Kreger, 1983).

Likewise, the extracellular proteases from the human pathogen *V. cholerae* have been involved in virulence. These enzymes seem to be implicated in bacterial intestinal colonization. In addition, Crowther, *et al.*, (1987) reported that their proteolytic activity degrades the protective layer of epithelial mucus and consequently enhance the activity of cholera enterotoxin. Similar role in pathogenicity has been suggested for the extracellular proteases of *Vibrio mimicus* (Chowdhury, *et al.*, 1990), recognized as human pathogen causing some enteric infections, otitis media and wound infections. In agreement with those findings, protease-deficient mutants of *V. cholerae* have been shown to have decreased virulence (Schneider and Parker, 1978) which possibly reflects a decreased ability to colonize the gut (Booth, *et al.*, 1983). Decreased virulence of these mutants might also reflect deficiencies in the nicking and activation of the cholera toxin. The extracellular protease of *V. cholerae* has been found responsible for this activity (Booth, *et al.*, 1984).

Similar roles in amber disease might be speculated for the extracellular protease of *S. entomophila.* In addition to attacking and depressing the insect immune system, this enzyme might assist in the degradation of the gut membrane in collaboration with bacterial chitinases. This role of *S. entomophila* chitinases in amber disease has been previously suggested (Glare, *et al.*, 1992). *S. entomophila* invades the larvae haemocoel during the late periods of infection, breaking through the foregut membrane, which contains chitin and lipoproteins.

A toxin protein has been suggested involved in amber disease (Chapter VI). Some toxic proteins need to be processed to be active, including the cholera toxin, diphtheria toxin, Shiga toxin, exotoxin A of *P. aeruginosa* (Middlebrook and Dorland, 1984) and the δ-endotoxin of *B. thuringiensis* (Höfte and Whiteley, 1989). The extracellular protease of *V. cholerae* processes cholera toxin to its active form (Booth,

et al., 1984). The extracellular protease of *S. entomophila* could share a similar function.

Reduced levels of protease activity were observed in UC57 and no apparent growth deficiencies were presented by this mutant. Besides, a single Tn*phoA* insertion in the bacterial genome was found (Manning, *et al.*, 1993). Hence, this mutant was selected to carry out complementation tests.

Plasmid pPRO1 containing genes encoding protease, reestablished the proteolytic activity in the mutant UC57 (Table 3.3). However, the size of halos produced by the mutant carrying the plasmid, were smaller than those observed in the parental strain BC4B (Table 3.3). On the other hand, pathogenicity was not restored by the protease genes (Fig. 3.5). Therefore, the mutant UC57 was not complemented by pPRO1 containing protease gene(s).

The mutant UC57 is apparently deficient also in chitinase activity (Table 3.3), which is an evidence of pleiotrophical effects of the Tn*phoA* insertion in UC57 genome. Mutants UC61 and UC7 showed also deficiencies in both protease and chitinase activities (Table 3.3). These features may be also related with pleiotropic mutations, although in the case of UC61, those deficiencies could be explained by the possibility of multiple insertions of Tn*phoA* in the genome of this mutant. Pleiotropic changes affecting other extracellular enzymes have been reported in protease-deficient mutants of *P. aeruginosa* (Wretlind, *et al.*, 1977). Besides, most protease-deficient mutants in *V. cholerae* had additional genetic lesions which could account for avirulence (Schneider and Parker, 1978).

An interrelation between extracellular enzymes and toxin(s) in *P. aeruginosa*, *V. cholerae* and *S. entomophila*, might determine the lack of virulence observed in protease deficient mutants.

Virulence genes in bacteria seem to be organized in regulons (see Chapter I). It is becoming evident that different regulons relate to each other by overlapping networks forming a very complex gene regulatory system. An interesting example is the cAMP receptor protein (CRP). This regulator is essential in virulence of *S. typhimurium* (Curtiss and Kelly, 1987) and controls and links at least 200 genes (Dorman and Bhriain, 1992). As it was previously mentioned, proteases from pathogenic bacteria probably share a common regulator with other virulence genes.

Catabolite repression[3] has been reported as a regulatory factor of *S. marcescens* proteases (Bromke and Hammel, 1979), therefore cAMP might be a link of proteases with other virulence genes.

Failure of the protease genes to restore pathogenicity in UC57 in the complementation assay suggests: i) that this enzyme do not play a direct role in development of amber disease and consequently the Path$^-$ character of UC57 is associated with, but not caused by, deficiencies of protease activity; ii) that deficiencies in protease activity in UC57 apparently are linked with virulence determinants probably by means of a common regulatory network and iii) insertion of Tn*phoA* in UC57 might be disrupting a "master" regulator gene or some other unknown factors essential for pathogenicity. A global regulator, named LasR has been shown required for the transcription of elastase genes (*lasA* and *lasB*) in the opportunistic human pathogen *P. aeruginosa* (Passador *et al.*, 1993). The genes *lasA* and *lasB* encode extracellular proteases involved in pathogenicity. The product of the *lasR* gene also controls the expression of other virulence factors as the alkaline protease and the toxin A of *P. aeruginosa*. Therefore, is not surprising the existence of global regulators controlling virulence factors, including the protease genes in *S. entomophila*. On the other hand, even though there is no clear evidence on an active role of *S. entomophila* chitinases on amber disease, deficiencies of chitinase activity shown by UC57 (Table 3.3) might account for its Path$^-$ phenotype and/or the failure of the protease genes to restore pathogenicity.

Proteases from several bacteria species have been cloned and sequenced. Strong homology among them has been reported, specially in areas regarded to contain a zinc ligand and the active site. These species include *V. cholerae* (Häse and Finkelstein, 1991), *Erwinia chrysanthemi* (Dahler, *et al.*, 1990), *P. aeruginosa*, *Bacillus thermoproteolyticus*, *Bacillus subtilis* and *Serratia* sp. (Bever and Iglewski, 1988; Nakahama, *et al.*, 1986). Hence, a common function of protease in pathogenicity might be speculated. Although proteases are also present in nonpathogenic bacteria, these proteolytic enzymes might bring advantages to virulent microorganisms.

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[3] Catabolite repression describes the decreased expression of many bacterial operons that results from addition of glucose. It is caused by a decrease in the level of cyclic AMP, which in turn inactivates the CRP (CAP) regulator. This is needed for RNA polymerase to initiate transcription of the catabolite-sensitive operons in E. coli (Lewin, 1987).

In summary, these results suggest that the extracellular protease of *S. entomophila* is not directly related with amber disease symptoms. The enzyme might be linked to virulence determinants by a regulatory factor. However, an active role in the general mechanism of pathogenicity to potentiate the disease is not discarded, since loss of pathogenicity in some mutants of the Path$^+$ strain UC9 correlated with deficiencies in protease expression after Tn*phoA* insertion mutagenesis (Table 3.3). Therefore, three functions might be hypothesized for *S. entomophila* extracellular protease in amber disease: i) as a factor attacking the insect immune system; ii) as a factor degrading the protective gut membrane to render bacteria access into the insect haemocoel and iii) as a factor processing a precursor protein into an active toxin involved in the disease (see Chapter IV). If any of these functions were proved, an active role for proteases in pathogenicity would be established.

Finally, the cloning of the extracellular protease of *S. entomophila* provides the molecular base to construct true protease mutants, specifically in the enzyme structural gene(s). This approach would be very useful to determine the role of proteases in amber disease.

Further analysis of the other nonpathogenic mutants generated in this work should help our understanding of the regulation of pathogenicity and the role of proteases in amber disease.

CHAPTER IV

IDENTIFICATION, ISOLATION AND CLONING OF *amb2* LOCUS FROM *Serratia* entomophila: GENES CONFERRING ANTI-FEEDING EFFECT ON LARVAE OF *Costelytra zealandica*

1) INTRODUCTION

Research in molecular genetics of amber disease caused by *S. entomophila* in the grass grub *C. zealandica*, has been concentrated on the study of the genetic loci involved in bacterial pathogenicity (Corbett, 1990; Shum, 1992; Upadhyaya, *et al.*, 1992). The disease presents two main symptoms: cessation of feeding (anti-feeding effect) and development of amber coloration. This chapter describes the identification, isolation and cloning of a *S. entomophila* genetic locus associated with amber disease and conferring anti-feeding effect to the infected larvae.

2) MATERIALS AND METHODS

2.1) Bacterial strains and vectors

The bacterial strains and plasmid vectors for cloning utilized in this work are listed in Tables 4.1 and 4.2. Media and growth conditions are described in Chapter 2, section 2.1. The protocol for preparing milk and chitinase agar plates is described in Chapter III, section 2.3. *E. coli* HB101 and DH5α were used for transformation recipients and hosts for the maintenance of recombinant plasmids when α-complementation of ß-galactosidase was required. Bacterial strains were maintained at -80°C in LB medium containing 25% (vol/vol) glycerol.

2.2) DNA procedures, restriction mapping and cloning

General genetic methods were performed as previously described (see Chapter III, section 2.6). Transfer of plasmids to *S. entomophila* was carried out either, by tripartite conjugation or by electroporation (see Chapter III, section 2.7). The plasmid from *S. entomophila* UC9 was purified as reported by Comai and Kosuge (1982), which is a method suitable for megaplasmids. Briefly, bacteria from 25 ml

Table 4.1. Bacterial strains used in this study

Strain	Relevant Characteristics	Reference
S. entomophila		
A1MO2	Wild-type; Path$^+$	T.A. Jackson
UC9	Clonal selection from A1MO2;Ap^r Path$^+$	H.K. Mahanty
UC7	Spontaneous Km^r mutant from A1MO2; Ap^r Km^r Path$^-$	H.K. Mahanty
UC24	Tn*phoA* mutant derivative from UC9; Path$^-$ Ap^r Km^r Gm^r	195
UC21	Tn*phoA* mutant derivative from UC9; Path$^-$ Ap^r Km^r Gm^r	195
UC57	Tn*phoA* mutant derivative from BC4B; Ap^r Km^r Gm^r	H.K. Mahanty
A20	Wild-type; Path$^-$	83
E. coli		
HB101	F$^-$ *pro leu thi lacY recA hsdR hsdM*	166
DH5α	F$^-$ *lacZ* M15 *endA1 hsdR17 supE44 thi-1 gyrA relA1*	171
JM83	lambda- *ara (pro-lac)rpsL thi* ϕ*80dlacZ* M15lambda-	166

of an overnight culture were centrifuged for 10 min (6K, Sorvall, rotor SS34) and resuspended in 0.5 ml of 15 mM NaCl. Bacteria were lysed by adding 12.5 ml of 3% SDS in TEN buffer (10 mM Tris, 10 mM NaCl, 1 mM EDTA [pH 9]). The lysate was incubated at 50°C for 10 min and adjusted to pH 8-9 with 2 M Tris (pH 5.5). The preparation was adjusted to 1 M NaCl adding 3.25 ml of 5M NaCl and incubated on ice for 1 h. The lysate was centrifuged at 12,000 x g (4°C) for 15 min. Four ml of 50% polyethylene glycol 6000 in 10 mM Tris (pH 8) were added to the supernatant containing the plasmid. The sample was incubated on ice for 1 h and centrifuged at 5,000 x g (4°C) for 10 min. The supernatant was discarded and the pellet was resuspended in 200 μl of TES buffer (50 mM Tris [pH 8.0], 30 mM NaCl, 5 mM EDTA). The plasmid preparation was further purified by phenol extraction and ethanol precipitation.

2.2.1) Tripartite conjugation assays

Table 4.2. Plasmids used in this study

Plasmid	Relevant Characteristics	Reference
pBR322	ColE1 *ori* Ap^r Tc^r; cloning vector; 4.36 Kb	15
pUC18	ColE1 *ori lacZ* Ap^r; cloning vector; 2.69 Kb	200
pLAFR3	pRK290 derivative with lambda *cos lacZ* and polycloning sites of pUC9; RP4 (IncP-1) *ori* Tc^r; cloning vector; 22 Kb	184
pRK2013	ColE1::pKK2 IncP-1 *tra* Km^r	51
pSER4	9 Kb *Bam*HI insert containing part of Tn*phoA* and flanking *S. entomophila* DNA from UC24 cloned into pBR322; Ap^r Km^r	H.K. Mahanty
pCOS1	pLAFR3 cosmid clone from *S. entomophila* UC9 library with a 21.3 Kb DNA insert containing *amb2*; Tc^r	This work
pCOS13	Similar to pCOS1 but with 24.6 Kb DNA insert	This work
pCOS31	Similar to pCOS1 but with 29.7 Kb DNA insert	This work
pCOS33	Similar to pCOS1 but with 27 Kb DNA insert	This work
pCOS35	Similar to pCOS1 but with 24 Kb DNA insert	This work
pCOS36	Similar to pCOS1 but with 25.6 Kb DNA insert	This work
pENV1	*Bam*HI insert fragment of 5.3 Kb containing *amb2* from pCOS31 cloned in pLAFR3; Tc^r	This work
pENV2	*Bam*HI insert fragment of 5.3 Kb containing *amb2* from pENV1 cloned in pUC18; Ap^r	This work
pSER201A	pBR322 with 8.6 Kb *Bam*HI fragment containing *amb1*; Ap^r	195

Conjugation for transfer of plasmids from *E. coli* HB101 to *S. entomophila* strains was performed due to the failure of *S. entomophila* transformation by conventional methods. Because of the lack of *mob* sequences in plasmids arising from pLAFR3, we used *E. coli* carrying pRK2013 as the helper plasmid. Tripartite conjugation was performed basically as described (Ditta, *et al.*, 1980). Briefly, broth cultures grown overnight in the presence of suitable antibiotics, were mixed in microcentrifuge tubes in the following proportion:

S. entomophila	750 µl
E. coli (carrying pLAFR3)	500 µl
E. coli (carrying pRK2013)	500 µl

The mixture was centrifuged 2 min at 6K rpm and resuspended in 300 µl of LB broth with no antibiotics. The bacteria were incubated 60 min at 30°C. Transconjugants were selected by resistance to different antibiotics. Colonies were isolated and purified twice on plates with antibiotics. After confirmation of the presence and stability of the plasmids in *S. entomophila*, the transconjugants were used for bioassays.

2.3) Hybridization analysis

The 3.6 Kb fragment from pSER4 was purified after agarose gel electrophoresis. The fragment was used for preparing ^{32}P labelled probe by the random priming oligolabelling reaction (Sambrook, *et al.*, 1989; see next page). The *S. entomophila* cosmid library (Upadhyaya, *et al.*, 1992) was spread on selective medium (Tc) and grown overnight. Next, twelve hundred colonies were blotted onto nylon filters (Hybond-N, Amersham, International LTD, England) according to manufacturer's recommendations to be hybridized to the 3.6 Kb radioactive probe.

Cosmid DNA was isolated and digested with restriction enzymes. A 0.7% agarose gel electrophoresis was run at 25V overnight. The DNA was then Southern blotted onto nylon filters as mentioned above and hybridized to the radioactive probe.

2.3.1) Radioactive probes

DNA radioactive probes were prepared with the Multiprime DNA Labelling System (RPN.1601Z, Amersham). The DNA fragments for preparing the probes were

recovered from agarose gels by electroelution on DEAE membrane. Briefly, the gel was cut with scalpel in front of the desired bands and the DEAE filter was placed into the slit. The slit was closed and the gel was run 2 min at 200 V. The filter was rinsed with TE buffer (10 mM Tris-HCl pH 8, 1mM EDTA) and incubated at 65°C in 400 µl of elution buffer (1M NaCl, 0.05 M arginine). After 2 hrs, the filter was discarded and 0.9 ml of 100% ethanol were added to precipitate the DNA. The samples were placed at -20°C overnight before centrifugation.

The recovered DNA (~250 ng), was digested with *HaeIII* (1-2 units) for 20 min at 37°C. Ten µl of H_2O were added for a total vol. of 30 µl. The samples were boiled 2 min and warmed at 37°C. The following mixture was prepared:

DNA	30.0 µl
Sol1 (kit)	10.0 µl
Sol2 (kit)	5.0 µl
[α-^{32}P]dCTP	2.5 µl
DNA polymerase I (Klenow fragment)	2.0 µl (2 units)

The reaction mixture was incubated at room temperature (RT) overnight.

The unincorporated label was removed by passing the sample through a Sephadex-G50 (Pharmacia) column, prepared in a 1 ml disposable syringe. The effluent containing the probe was stored at -20°C.

2.3.2) Colony Hybridization

The colony hybridization technique involves the transfer and lysis of the colonies onto a nylon membrane. The DNA is denatured *in situ* and fixed onto the screen. This membrane is hybridized with a radioactively labelled probe that represents partially the desired sequence to be located in the library. Any colonies that have hybridized to the probe are visualized as dark spots by autoradiography onto X-ray film. The corresponding colonies can be recovered from the agar plates containing the original reference library.

Colonies were grown overnight on LB-agar and the nylon membranes were placed on the surface of the plates. After 10 min, the membranes were removed and

put onto 3MM filter paper. The membranes were soaked once with denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 7 min and twice with neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl pH 7.2, 0.001 M EDTA) for 3 min. Then, the membranes were briefly washed in 2X SSC solution (20X SSC: 175.3 g/lt of NaCl, 88.2 g/lt of Sodium citrate, adjusted to pH 7). To fix the DNA, the membranes were soaked for 30 min in 0.4 M NaOH and finally rinsed with 5X SSC solution. The membranes were stored in plastic bags at 4°C.

Pre-hybridization was carried out at 65°C for 2 hrs in 25 ml of hybridization buffer (2.5 ml of 20X SSC, 2.5 ml of 50 X Denhardts, 0.315 ml of Sheared herring sperm DNA [10 mg/ml], 20 ml H_2O). Eighty per cent of the buffer was poured out and the radioactive DNA probe, previously denatured by boiling, was added (2-5 µl, ~ 1000 cpm). Hybridization was performed overnight at 65°C. The membrane discs were washed twice at room temperature for 5 min in 2X SSC solution.

2.3.3) Southern blot hybridization

DNA from agarose gels was transfered to nylon membranes in a Blot Transfer Apparatus (VacuGene^{TM}, Pharmacia LKB), basically as suggested by the manufacturers. DNA was depurinated by adding 50 ml of 0.25 N HCl on top of the gel. The HCl was left until the bromophenol blue tracking dye turned yellow, approximately 15 min. After pouring out the HCl, the DNA was denatured by adding 50-100 ml of 0.4 M NaOH. The gels were soaked in NaOH for 90 min on the Blot Transfer Apparatus. Membranes were dried onto paper towels and stored at 4°C.

The membranes were pre-hybridized for 2 hrs at 65°C in Southern hybridization buffer (1mM EDTA, 0.5M Na_2HPO_4 pH 7, 7% SDS). The labelled probe was added (2-5 µl, ~2000 cpm) and the membranes were incubated overnight at 65°C for hybridization. Finally, the membranes were soaked for 30 min in washing solution 1 (1mM EDTA, 40 mM Na_2HPO_4 pH 7, 5% SDS). A second washing was performed for 60 min in solution 2 (1mM EDTA, 40 mM Na_2HPO_4 pH 7, 1% SDS).

2.4) Analysis of fimbriae (haemagglutination tests)

Media, culture conditions and the method for determination of haemagglutination activities are described in Chapter II, section 2.2, 2.3 and 2.4.

2.5) Preparation of supernatants of *S. entomophila*.

S. entomophila strains were grown overnight in LB broth. The cultures were centrifuged to pellet the cells (6 K, Sorvall SS34 rotor). The supernatants were filter sterilized and dialysed against H_2O for 48 hrs in a dialysis tube with an exclusion volumen of 10 kDa MW. After dialysis, the samples were again filter sterilized to avoid contamination and stored at -80°C. The samples were concentrated by lyophilization and resuspended in H_2O. Supernatants were concentrated 300 times.

2.6) Bioassays with grass grub larvae

Pathogenicity tests were performed basically as described previously (Upadhyaya, *et al.*, 1992). Unless otherwise specified, the tests were routinely carried out twice with 20 field-collected *C. zealandica* 3rd instar larvae. For some assays 2nd instar larvae were used. The larvae were maintained individually and fed with small pieces of carrot, approximately $8mm^3$ each. Those larvae that were actively feeding and looking healthy by visual observation were used for the tests. Each piece of carrot was impregnated with the test bacteria by rolling it onto LB plates (plus antibiotics if required) containing a lawn of overnight culture. Only one inoculation of approximately 10^8 bacterium per larva was applied in each experiment. Pathogen free carrot was used to feed the control larvae. Positive controls were routinely performed with the $Path^+$ strain UC9 and negative controls with nonpath strains (e. g. UC7; Fig. 4.1). Larvae were maintained at 15°C and were examined after 3,7,10,14 and 19 days from the beginning of the tests. At the same time, larvae were transferred to clean trays with uninoculated pieces of carrot. Visual observation was made on the amount of carrot consumed and the development of amber coloration. Non-feeding behaviour was considered when larvae consumed less than 50% of the carrot. Anti-feeding effect (AFE) was defined as the percentage of larvae showing non-feeding behaviour during the bioassays. Amber color (AC) is depicted as the percentage of larvae developing this symptom. AFE and AC percentages reported in the text and figures, represent maximum rates observed in the shortest period since inoculation. Net rates represent the arithmetic difference between AFE percentages of the test larvae and the negative control larvae.

Differences in the number of larvae showing amber disease symptoms under different treatments were assessed by hypothesis test in two-way frequency tables (contingency tables) using the Chi-squared (X^2) statistical test.

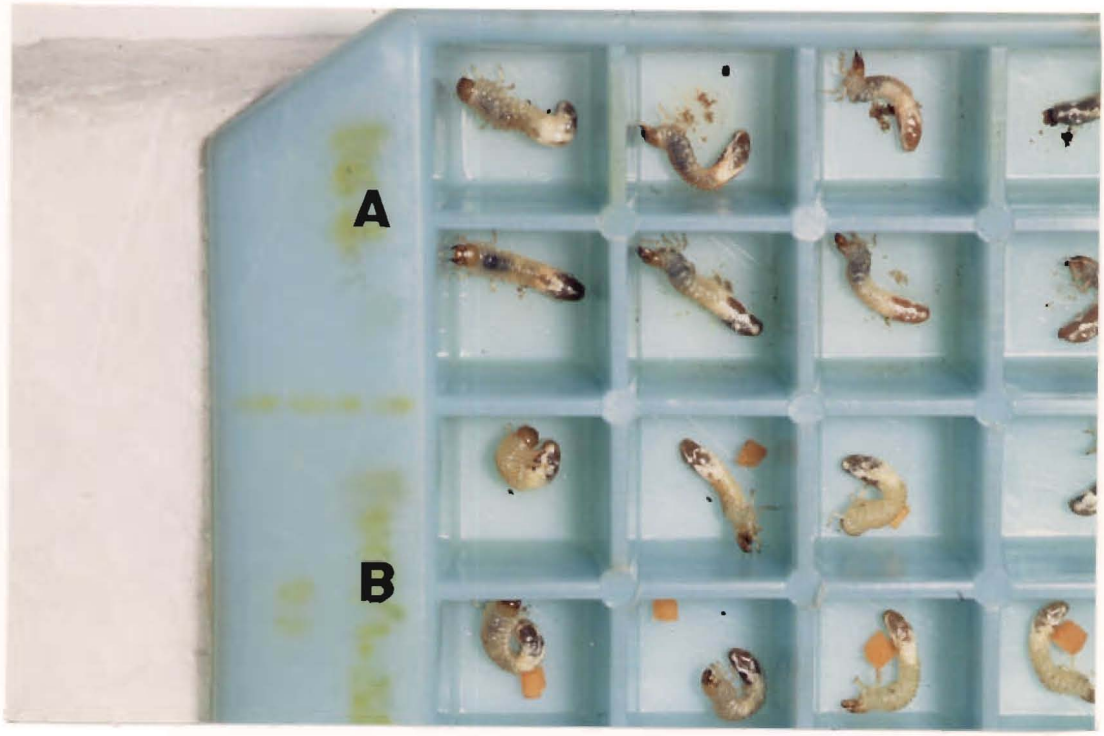


Fig. 4.1. Bioassays with *C. zealandica* larvae for pathogenicity tests. Larvae were fed with small pieces of carrot coated with suitable bacteria. One inoculation of approximately 10^8 cells per larvae was applied. Positive and negative controls were routinely performed with the pathogenic ($Path^+$) strain UC9 and the corresponding nonpathogenic ($Path^-$) strains. A) Healthy larvae fed with the $Path^-$ strain UC7 after 7 days from inoculation; B) Larvae fed with UC9 after 7 days from inoculation showing amber disease symptoms: anti-feeding effect and development of amber coloration (see Materials and Methods for details).

3) RESULTS

3.1) Identification, isolation and mapping of recombinant cosmids carrying genes for anti-feeding effect

Several nonpathogenic *S. entomophila* mutants were obtained in our laboratory by transposon (Tn*phoA*) mutagenesis (Upadhyaya, *et al.*, 1992). The mutant *S. entomophila* UC24 was selected by its failure to cause amber disease symptoms. Southern analysis of the mutant DNA revealed that UC24 had a single Tn*phoA* insertion in the bacterial genome. The Tn*phoA* insertion site was cloned into the *Bam*HI sites of pBR322, resulting in a plasmid carrying the DNA fragment of UC24 flanking the left end of Tn*phoA* (Fig. 4.2). The recombinant cosmid was named pSER4 (H. K. Mahanty, unpublished).

A *S. entomophila* cosmid library, (strain AIMO2) previously constructed by Upadhyaya, *et al.*, (1992), was screened for wild type homologous sequences to pSER4. To isolate the DNA fragment from this plasmid, it was previously digested with *Eco*RI. Then, partial digestion with *Hind*III was carried out to isolate the 3.6 Kb DNA fragment corresponding to sequences from the nonpathogenic ($Path^-$) UC24 mutant (Fig. 4.2). Six colonies showed homology to the radioactive probe. To confirm the presence of homologous sequences, cosmid DNA was isolated from these positive colonies and restricted with *Bam*HI and double digested with *Eco*RI and *Hind*III enzymes. After agarose gel electrophoresis the DNA was Southern blotted and hybridized with the same probe.

Hybridization was evident with the six isolated cosmids (Fig.4.3) confirming the presence of wild type *S. entomophila* DNA sequences. A single *Bam*HI 5.3 DNA fragment homologous to the probe, was present in four cosmids (Fig. 4.3, lanes A, E, G and I) showing the presence of an overlapping fragment. Several other *Eco*RI-*Hind*III fragments showed hybridization with the probe (Fig.4.3, lanes B, D, F, H, J and L) including an expected 1.3 Kb *Hind*III fragment previously mapped on pSER4 (H. K. Mahanty, unpublished). Other fragments that are not homologous to the probe were present in the digested DNA. The general restriction pattern in all the cosmids was different. A restriction map of the recombinant cosmids was deduced from restriction enzyme digestion data and from the Southern hybridization (Fig. 4.4). The estimated size of the DNA inserts was 21 to 30 Kb. Two cosmids, 31 and 35 cover a fragment of ~35.3 Kb of wild type DNA of *S. entomophila*. These cosmids

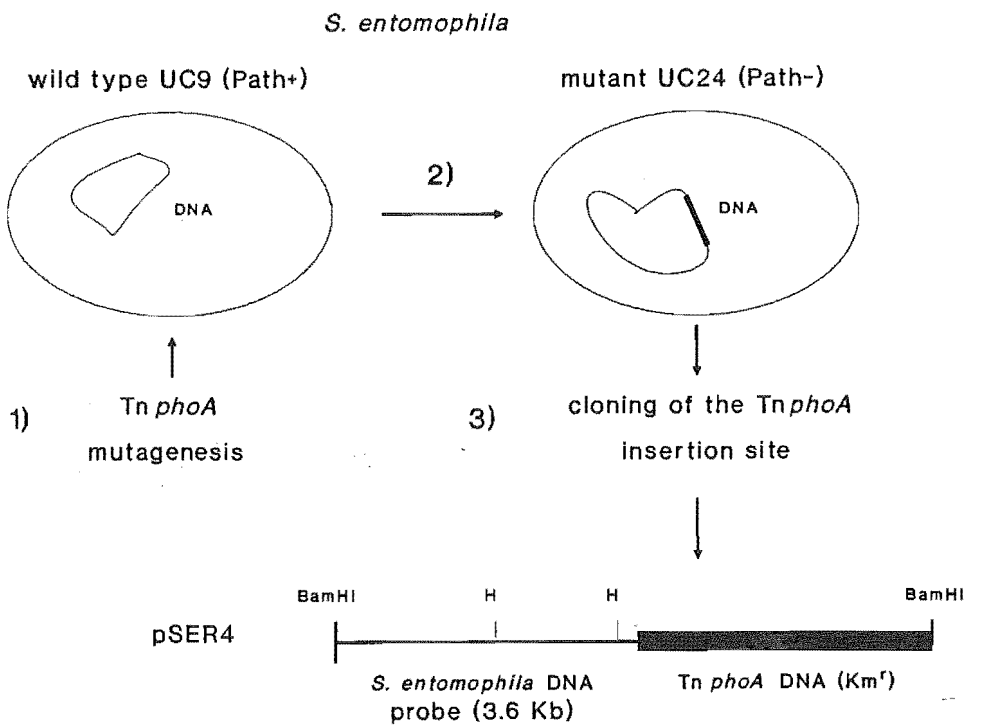


Fig. 4.2. Strategy for identification and cloning of the mutant locus in UC24. 1) Tn*phoA* mutagenesis was performed in the pathogenic *S. entomophila* strain UC9; 2) The mutant UC24 was selected for loss of pathogenicity by bioassays (Upadhyaya, *et al.*, 1992); 3) DNA from UC24 was digested with *Bam*HI and ligated with pBR322, also digested with *Bam*HI. Recombinant plasmids were selected for Ap^r (marker of pBR322) and Km^r (marker of Tn*phoA*) and designated pSER4 (H. K. Mahanty, unpublished).

Fig. 4.3. Southern blot analysis of cosmids isolated from the *S. entomophila* gene library containing *amb2* locus. Cosmid DNA was digested with *Bam*HI and double digested with *Eco*RI-*Hind*III. Hybridization was carried out with a ^{32}P labelled probe from the 3.6 Kb DNA fragment of pSER4 (Fig. 4.2). The 3.6 Kb fragment is located flanking the left end of the Tn*phoA* insertion site in mutant UC24 (Fig. 4.2). Lambda-*Hind*III markers are shown at the left. Lanes: A) pCOS1/*Bam*HI; B) pCOS1/*Eco*RI-*Hind*III; C) pCOS13/*Bam*HI; D) pCOS13/*Eco*RI-*Hind*III; E) pCOS31/*Bam*HI; F) pCOS31/*Eco*RI-*Hind*III; G) pCOS35/*Bam*HI; H) pCOS35/*Eco*RI-*Hind*III; I) pCOS33/*Bam*HI; J) pCOS33/*Eco*RI-*Hind*III; K) pCOS36/*Bam*HI; L) pCOS36/*Eco*RI-*Hind*III.

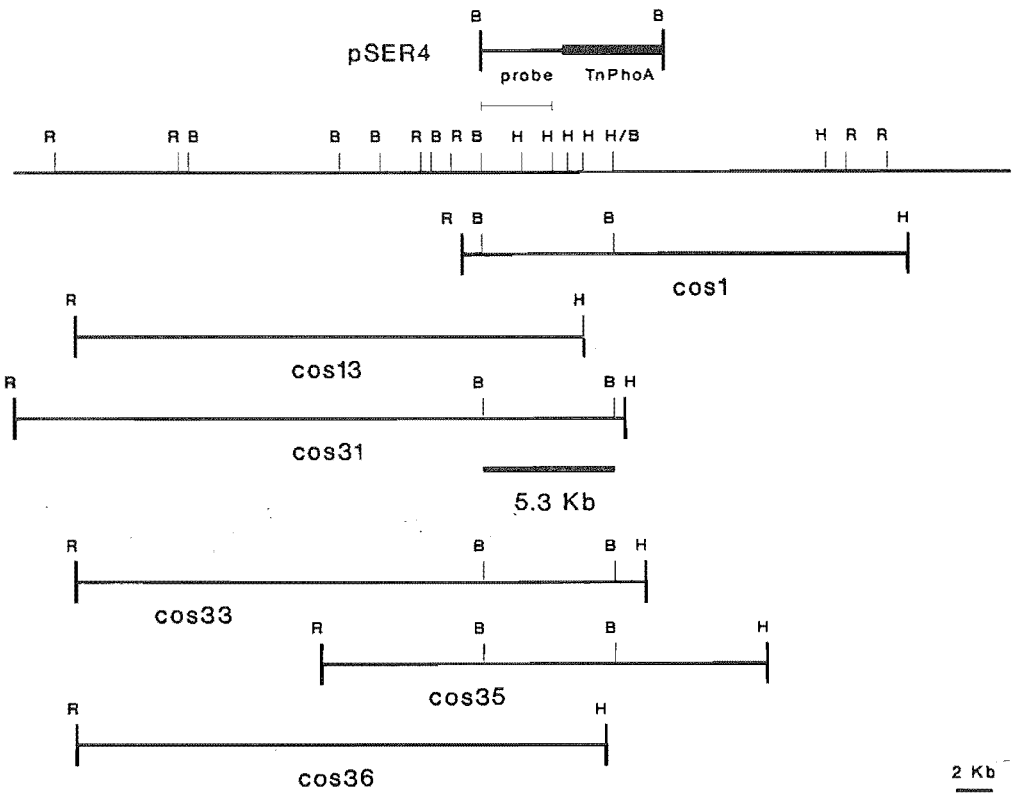


Fig.4.4. Restriction map of the cosmids isolated from the *S. entomophila* gene library carrying *amb2* locus. The map was deduced from *Eco*RI (R), *Hind*III (H) and *Bam*HI (B) digestion data and from the Southern hybridization shown in Fig. 4.3. A ^{32}P labelled probe from the 3.6 Kb fragment of pSER4, located upstream of the Tn*phoA* insertion in mutant UC24, was used to identify the cosmids by colony hybridization. The cosmids 31 and 35 were tested for complementation of pathogenicity in UC24 and fully restored the anti-feeding effect (AFE) in bioassays. The 5.3 Kb overlapping DNA fragment was subcloned and shown to be responsible of the AFE. The locus was named *amb2*.

apparently contain the DNA sequences of the other three cosmids 13, 33 and 36. The cosmid 1 covers an extra DNA fragment of 6Kb downstream of the right end of cosmid 35. The overlapping *Bam*HI DNA region of 5.3 Kb is present in the cosmids 1, 31, 33 and 35. Cosmids 13 and 36 apparently have only part of this region, since hybridization is evident with DNA still attached to the vector (Fig. 4.3., lanes C and K) and the 5.3 Kb DNA fragment was not observed when the cosmids were digested with *Bam*HI. The insertion site of Tn*phoA* was mapped in this overlapping fragment.

3.2) Complementation assay of nonpathogenic UC24 with cosmids carrying wild type DNA sequences

To confirm the relationship of pathogenic determinants with the DNA inserts present in the selected cosmids, complementation assays were carried out. These assays were performed with the cosmids 31 and 35, which have the largest fragment of wild type *S. entomophila* DNA, including sequences present in the cosmids 13, 33 and 36 and also the 5.3 overlapping fragment. Cosmid 1 usually gave low yields of plasmid DNA and was excluded from complementation assays.

The cosmids 31, 35 and pLAFR3 were transferred to the nonpathogenic mutant UC24 by conjugation as is detailed in Materials and Methods.

The presence and stability of cosmids within the cells, was confirmed by cosmid DNA preparation directly from the *S. entomophila* transconjugants and further transformation with the isolated DNA to *E. coli* HB101 . Restriction analysis of the plasmid content of HB101 transformants indicated that the different cosmids have not suffered any apparent structural rearrangement as compared to the same cosmid DNA extracted from *S. entomophila* library in *E. coli* colonies (results not shown).

The mutant UC24 carrying the cosmids were phenotypically characterized to test for restoration of pathogenicity and for the effect of cosmids on the expression of virulence factors as pili, protease and chitinase.

3.2.1) Bioassays with grass grub larvae

Tests were made with *C. zealandica* larvae fed with small pieces of carrot as described in Materials and Methods. The carrot was impregnated with pathogenic

(UC9), nonpathogenic (UC24 bearing pLAFR3) and UC24 carrying cosmids 31 and 35. Uninoculated carrot was used to feed the control group of larvae. Observation was made on feeding behaviour of and amber coloration development in the larvae. Results of this experiment are summarized in Fig. 4.5.

Cessation of feeding and amber coloration was clearly observed in larvae fed on pathogenic strain UC9. Such symptoms were not observed on control and on larvae fed on nonpath strain UC24, but cessation of feeding was observed for larvae fed on carrot coated with UC24 carrying the cosmids 31 and 35. The percentage of larvae presenting anti-feeding effect (AFE) was 94.7 and 80 % respectively ($P<0.001$). This result was similar to the AFE observed in larvae fed with the pathogenic strain UC9 (81.3 %; $P<0.001$). Development of amber coloration was observed as well in larvae fed with UC24 bearing the recombinant cosmids, though to a lesser degree than UC9. The percentage of larvae showing amber color was 45 % for cosmid 31 and 30 % for cosmid 35 ($P<0.05$).

The results showed that the cosmids 31 and 35 fully restored the anti-feeding effect in the nonpathogenic mutant UC24. Also, the cosmids partially reestablished the ability to cause development of amber coloration. Therefore, it was confirmed that the *S. entomophila* wild type DNA sequences contained in the cosmids 31 and 35 were associated with amber disease.

The next step in the development of this research was to identify and clone the DNA fragment contained in the cosmids 31 and 35 associated with the disease. This DNA region was named *amb2* locus.

3.3) Cloning *amb2* locus into plasmid pLAFR3

The recombinant cosmids 31 and 35 contain *S. entomophila* DNA fragments of ~30 and 24 Kb. The Tn*phoA* insertion site causing loss of pathogenicity in the mutant UC24 was mapped in the wild type 5.3 Kb *Bam*HI cosmid overlapping fragment (Fig. 4.3 and 4.4). A map was deduced from the restriction pattern. Since amber disease symptoms were conferred by both cosmids 31 and 35, the rationale was that the overlapping DNA fragment contained the gene(s) responsible for such symptoms. In order to isolate and transfer by tripartite conjugation this segment of DNA to the UC24 mutant, the overlapping DNA fragment was first cloned into pLAFR3. Cosmid 31 was completely digested with *Bam*HI and the 5.3 Kb DNA

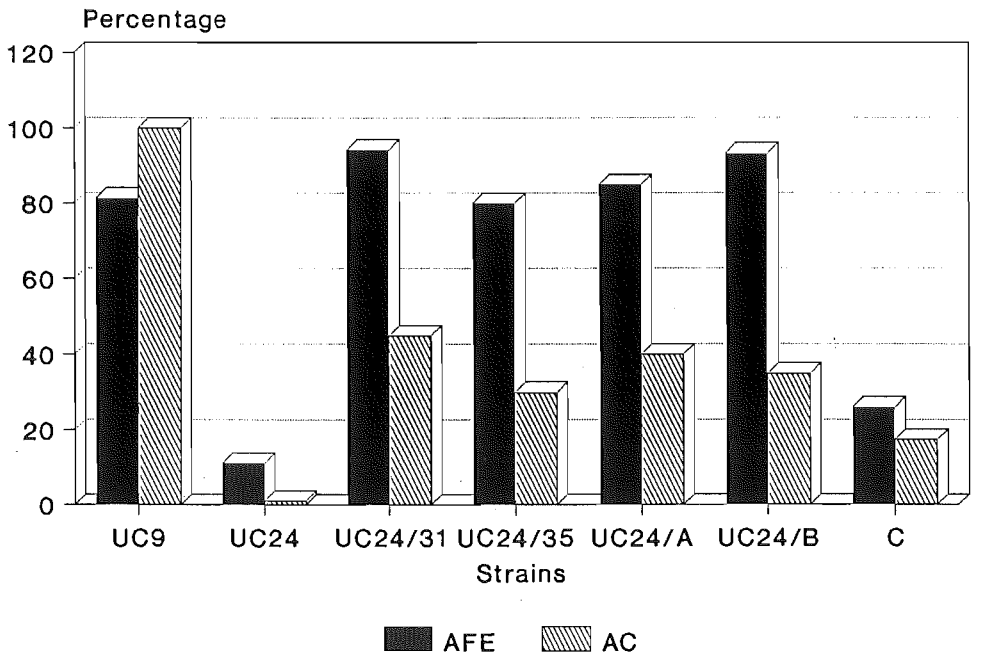


Fig. 4.5. Insect larvae bioassay of *S. entomophila* Path$^+$ UC9 and Path$^-$ UC24 carrying *amb2* locus. Cosmids 31 (pCOS31) and 35 (pCOS35) were rescued from the *S. entomophila* gene library and tested for restoration of pathogenicity in UC24. The overlapping 5.3 Kb DNA fragment was cloned in both orientations in pLAFR3 to create pENV1, OriA and OriB. Both plasmids were also transferred to UC24 to test for pathogenicity (UC24/A and UC24/B respectively). The positive control was UC9. UC24 carrying the vector pLAFR3 and uninoculated carrot were used as negative controls. Anti-feeding effect (AFE) and amber coloration (AC) are in percentage of larvae showing these amber disease symptoms after 7 days of treatment.

fragment was purified and ligated to the CIP treated *Bam*HI restricted vector pLAFR3. *E. coli* JM83 was transformed with the ligation mixture to yield the recombinant plasmid pENV1. White colonies growing on agar plates in presence of Tc and X-gal were examined for the presence of recombinant plasmid. *Bam*HI digestion of the plasmid DNA was carried out to confirm the expected plasmid. Both orientations of the 5.3 Kb fragment were obtained and named pENV1 OriA and pENV1 OriB (Fig. 4.6). This DNA was then transferred to the nonpath mutant UC24 by electroporation for further bioassay with larvae.

3.3.1) Bioassays with larvae and *amb2* Locus

In order to confirm that the 5.3 Kb DNA fragment was responsible for AFE and/or AC, UC24 carrying pENV1, OriA and OriB, were used for bioassays with larvae of *C. zealandica*. The results of this test are shown in Fig. 4.5. The rates observed were up to 85 % and 93.3 % of larvae showing anti-feeding effect with UC24 containing pENV1 OriA and OriB respectively. The effect was acute, as compared with 11.1 % of the control group fed with UC24 carrying the cosmid vector pLAFR3 ($P<0.001$). Development of amber coloration was also observed in larvae fed with UC24 carrying pENV1, with values up to 40 % and 35 % of the larvae tested for (OriA and OriB respectively; $P<0.05$). The rates of AFE and AC observed, were similar to those observed with the full cosmids 31 and 35. The orientation of the cloned DNA in pENV1 did not produce different results, confirming the integrity of the *amb2* locus in the plasmid pENV1.

3.4) General characterization of *amb2* locus

It was confirmed by bioassays that the *amb2* locus was responsible for the reestablishment of AFE and/or AC in the mutant UC24. However, it was unknown at this stage of the research whether *amb2* was restoring the deficiency of a regulatory gene in UC24 (e. g. a regulator controlling a virulence regulon; see Chapter I, section 2) or a gene encoding a virulence factor (e. g. pili, toxin). Pathogenic bacteria contain a full collection of virulence genes acting together to produce a disease. A mutation in one gene of the network may directly or indirectly cause loss of pathogenicity. Obviously, total lack of the genetic network would result in nonpathogenicity. On the other hand, amber disease symptoms may be restored partially or totally by a defined DNA locus depending upon its position in the genetic network and relationship with other pathogenic determinants. Accordingly, several

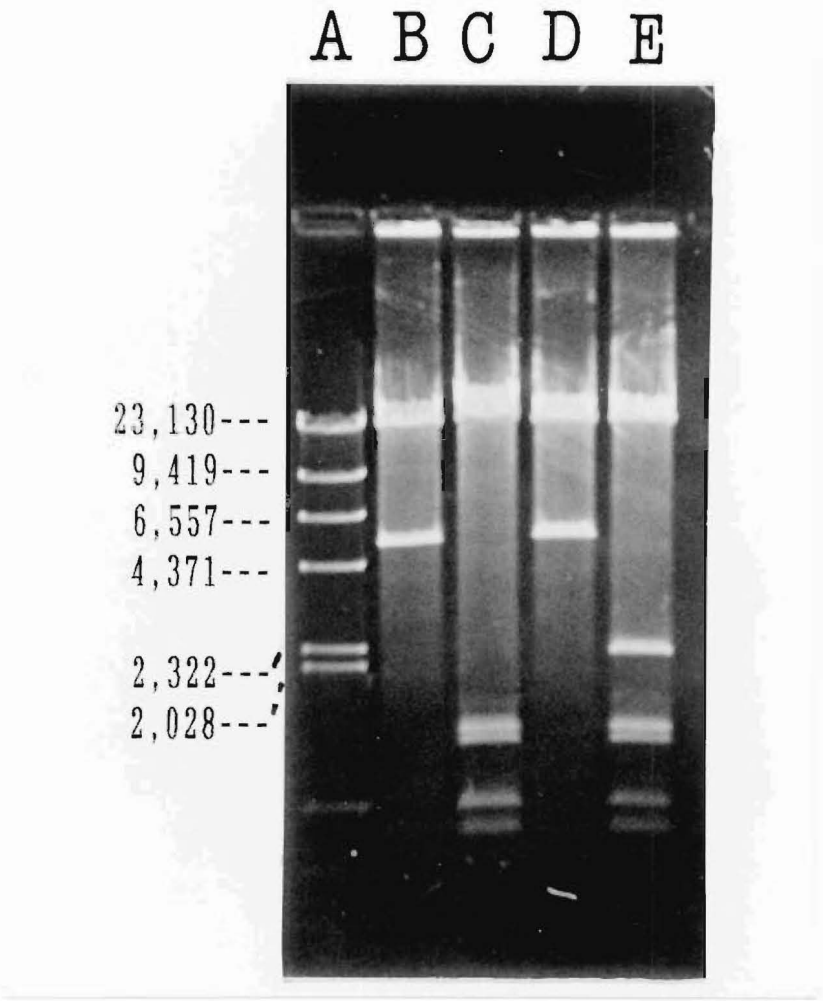


Fig. 4.6. Agarose gel electrophoresis of the 5.3 Kb DNA fragment cloned in pLAFR3. The 5.3 fragment was isolated from pCOS31 and ligated with *Bam*HI digested pLAFR3. Both orientations of the insert were obtained; the plasmids were named pENV1 OriA and pENV1 OriB. Lanes: A) Lambda/*Hind*III; B) pENV1 OriA/*Bam*HI; C) pENV1 OriA/*Hind*III; D) pENV1 OriB/*Bam*HI; E) pENV1 OriB/*Hind*III. 0.7% agarose gel.

nonpathogenic mutants were selected to analyze the effect of *amb2* locus on: i) amber disease symptoms; ii) fimbriae; iii) protease activity and iv) chitinase activity. The different strains used were selected on the basis of deficiencies observed on these virulence factors. Tables 4.1 and 4.4 shows the collection of mutants with different phenotypes.

3.4.1) Complementation assays of nonpathogenic mutants carrying amb2 locus

Cosmids 31, 35 and/or pENV1 were transferred, either by conjugation or electroporation, to the *S. entomophila* nonpathogenic strains UC7, UC21, UC57, and A20 for bioassays. Bacteria carrying pLAFR3 were used as controls. Results of the bioassays with insect larvae are summarized in Fig. 4.7, 4.8, 4.9, and Table 4.3.

Partial restoration in the ability to cause amber disease symptoms was observed in UC7 and UC21 carrying *amb2* locus. Larvae fed with these mutants showed net AFE rates of up to 88.2% (Fig. 4.7; $P<0.001$) and 38.7 % (Fig. 4.8; $P<0.05$) respectively. The net AFE displayed by larvae fed on UC57 was only 34.2% (Fig. 4.9), which was not statistically significant ($P>0.05$). Development of amber coloration was caused only by UC7 bearing pENV1 with AC of up to 50.7 % (Fig. 4.7; $P<0.01$). No symptoms were observed in larvae fed with A20 carrying pENV1 (Table 4.3).

3.4.2) Analysis of fimbriae, chitinase and protease expression in *S. entomophila* strains carrying *amb2*

Strains of *S. entomophila* UC9, UC24, UC21 and UC7 carrying cosmid 31, 35 and/or pENV1 were analyzed for expression of MRE-HA fimbriae, chitinase and protease as specified in Materials and Methods. *E. coli* HB101 carrying *amb2* locus was analyzed as well. The same isolates carrying the vector pLAFR3 were used in parallel as controls. Results are summarized in Table 4.4.

Apparent levels of chitinase and protease, measured as a size of the diameter of a halo on plates, were identical between strains carrying *amb2* and control groups. There were no differences in size of halo either when cosmids were placed into the wild type UC9 *S. entomophila*. These results showed no effect of *amb2* locus on chitinase and protease activity. The results were similar for haemagglutination tests

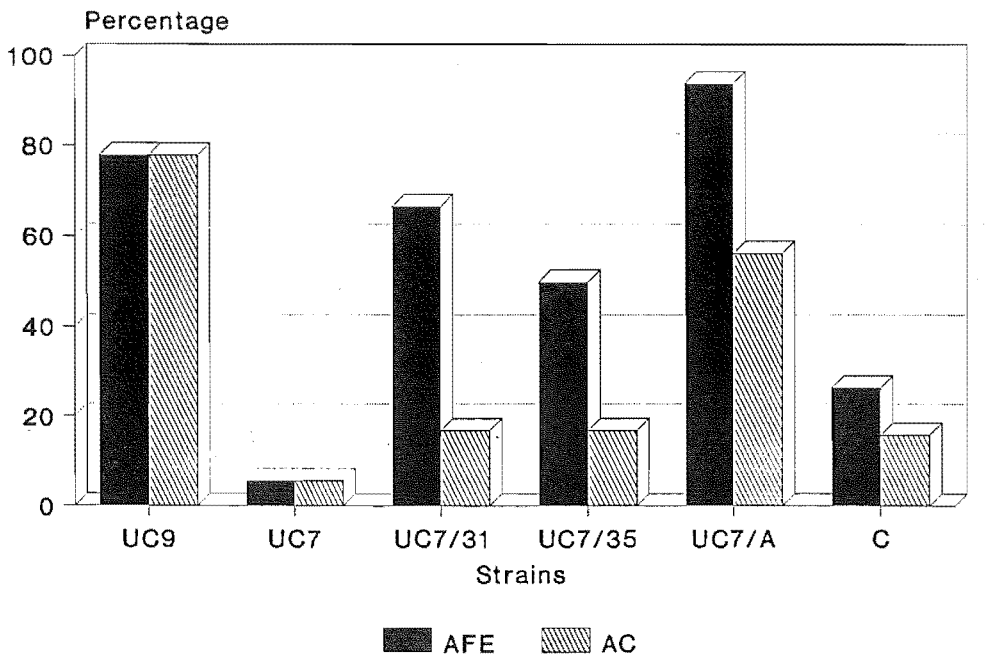


Fig. 4.7. Insect larvae bioassay of *S. entomophila* Path$^+$ UC9 and Path$^-$ UC7 carrying *amb2* locus. Plasmids pCOS31 (UC7/31), pCOS35 (UC7/35) and pENV1 OriA (UC7/A), were transferred to UC7 to be tested for complementation. The positive control was UC9. UC7 carrying the vector pLAFR3 and uninoculated carrot were used as negative controls. Anti-feeding effect (AFE) and amber coloration (AC) are in percentage of larvae showing these amber disease symptoms after 10 days of treatment.

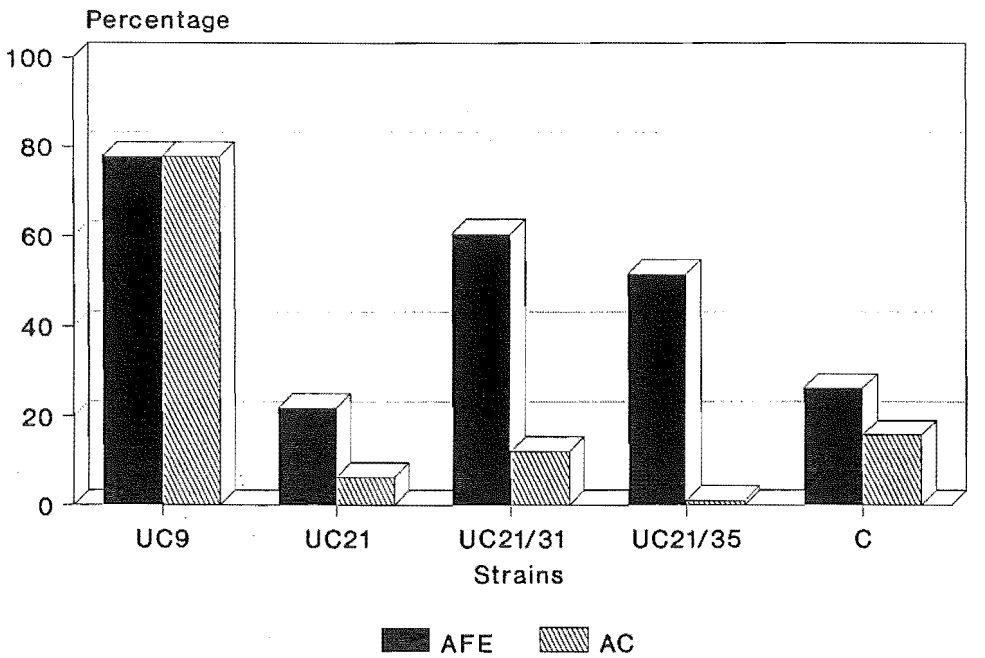


Fig. 4.8. Insect larvae bioassay of *S. entomophila* Path$^+$ UC9 and Path$^-$ UC21 carrying *amb2* locus. The mutant UC21 bearing pCOS31 (UC21/31) and pCOS35 (UC21/35) was tested for restoration of pathogenicity. The positive control was UC9. UC21 carrying the vector pLAFR3 and uninoculated carrot were used as negative controls. Anti-feeding effect (AFE) and amber coloration (AC) are in percentage of larvae showing these amber disease symptoms after 7 days of inoculation.

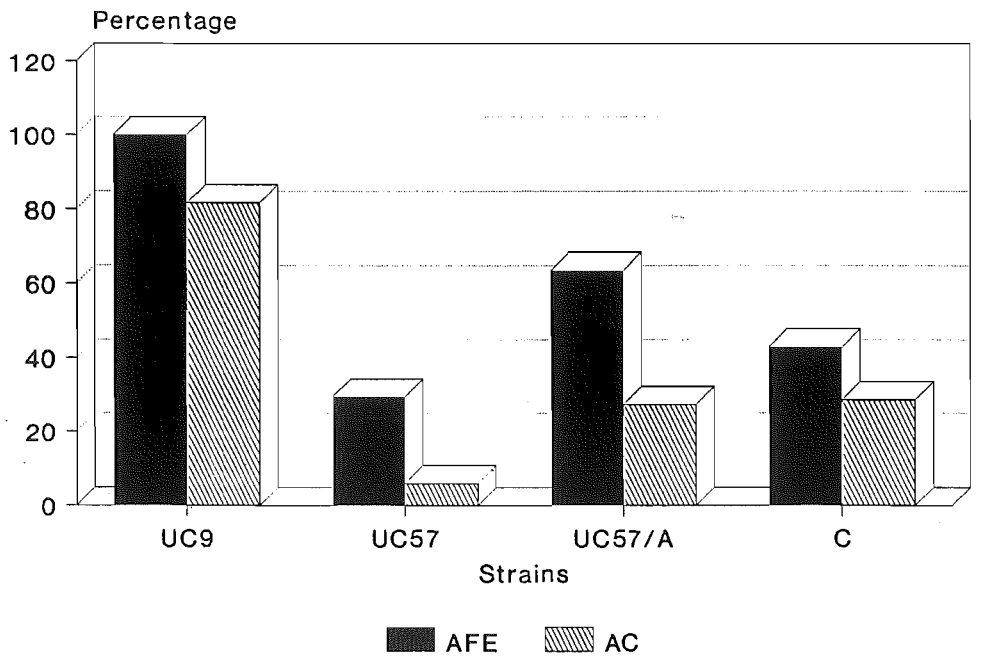


Fig. 4.9. Insect larvae bioassay of *S. entomophila* Path$^+$ UC9 and Path$^-$ UC57 carrying *amb2* locus. Plasmid pENV1 OriA was tested for restoration of pathogenicity in UC57 (UC57/A). The positive control was UC9. UC21 carrying the vector pLAFR3 and uninoculated carrot were used as negative controls. Anti-feeding effect (AFE) and amber coloration (AC) are in percentage of larvae showing these amber disease symptoms after 20 days of inoculation.

Table 4.3. Insect larvae bioassay of *S. entomophila* Path$^+$ UC9 and Path$^-$ A20 carrying *amb2* locus

Strain	AFE[a]	NET AFE[b]	AC
UC9[c]	76.9	50.20	69.2
A20/pLAFR3	26.7	0	0
A20/cos31	20	NA	0
A20/cos35	28.6	1.9	0

[a] Anti-feeding effect (AFE) and amber coloration (AC) are in percentage of larvae showing these symptoms after 7 days of inoculation.

[b] NET AFE represents the arithmetic difference between the AFE observed in each treatment and the AFE of the negative control.

[c] UC9 and A20 carrying pLAFR3 were used respectively as positive and negative controls.

Table 4.4. Phenotypic characterization of *S. entomophila* strains[ab]

Strain	Chitinases	Protease	MRE-HA
A1MO2	++++	++++	+
UC9	++++	++++	+
UC24	+++	+++	+
UC21	+++	++	-
UC57	++	+	+
UC7	+++	++	+
HB101	-	-	-
A20	++++	++++++	ND[c]
UC9/*amb2*	++++	++++	ND
UC24/*amb2*	+++	+++	+
UC21/*amb2*	+++	++	ND
UC7/*amb2*	+++	++	+
HB101/*amb2*	-	-	-

[a] Expression of fimbriae (MRE-HA), chitinases and protease activities was analyzed in pathogenic and nonpathogenic strains. The effect of *amb2* locus on these phenotypes was also analyzed. *E. coli* HB101 was used as negative control.

[b] Arbitrary degrees from - to ++++++.

[c] Not determined.

with bacterial suspensions of 5 x 10^7 to 5 x 10^{10} cells/ml. No change was detected on the mannose-resistant agglutination of erythrocytes (MRE-HA) when bacteria were carrying *amb2*. Since the MRE-HA is associated with the specific type of pili involved in amber disease (see Chapter III), *amb2* locus seems to have no effect either on the expression of this pathogenic determinant.

3.4.2.1) Lack of homology between *amb1* pili associated genes and *amb2* locus DNA

To date, two loci associated with amber disease have been isolated and cloned: *amb1* and *amb2*. Since pathogenic determinants seem to be interconnected and some of the cosmids containing *amb1* locus have been shown to cause cessation of feeding to larvae in the mutant UC21 (Upadhyaya, *et al.*, 1992) there was a chance that *amb1* locus had some homology to *amb2*. In order to eliminate this possibility a Southern blotting DNA hybridization test between *amb1* and *amb2* DNA was carried out. The plasmid pSER201A containing the *amb1* locus was *Bam*HI digested. The 9 Kb DNA fragment was isolated to make a radioactive ^{32}P probe. Apart, the plasmid pSER4 carrying sequences of *amb2* locus, was digested with *Hind*III for the Southern test. As it is shown in Fig. 4.10, no hybridization was detected between the DNA probe and pSER4 DNA. This results indicated that there was no homology between *amb1* and *amb2* DNAs and hence the possible similarity was excluded.

3.4.3) Origin of *amb2* locus: Plasmid or chromosome?

Results from the analysis of *amb2* locus suggested the possibility of a toxin activity encoded by *amb2* locus. This possibility raised the question about the location of this DNA. Several virulence factors from different origins and specificity are plasmid encoded, including the heat labile (LT) and heat stable (ST) enterotoxins of *E. coli* (Elwell and Shipley, 1980) and most of the insecticidal crystal proteins of *Bacillus thuringiensis* (Höfte and Whiteley, 1989). Besides, a *S. entomophila* plasmid of 105 Kb present in Path$^+$ strains has been associated with virulence (Glare, *et al.*, 1993a). Therefore, in order to elucidate the location of the *amb2* locus, a DNA hybridization test was carried out.

The plasmid pENV2 carrying *amb2* locus was *Bam*HI digested to isolate the 5.3 Kb DNA fragment for preparing the probe. Undigested plasmid from the Path$^+$ strain *S. entomophila* UC9 was used for the Southern blotting test. As it is shown in the Fig. 4.11., no hybridization was observed between both DNA sequences. This result strongly suggested that *amb2* locus did not lie in the *S. entomophila* plasmid.

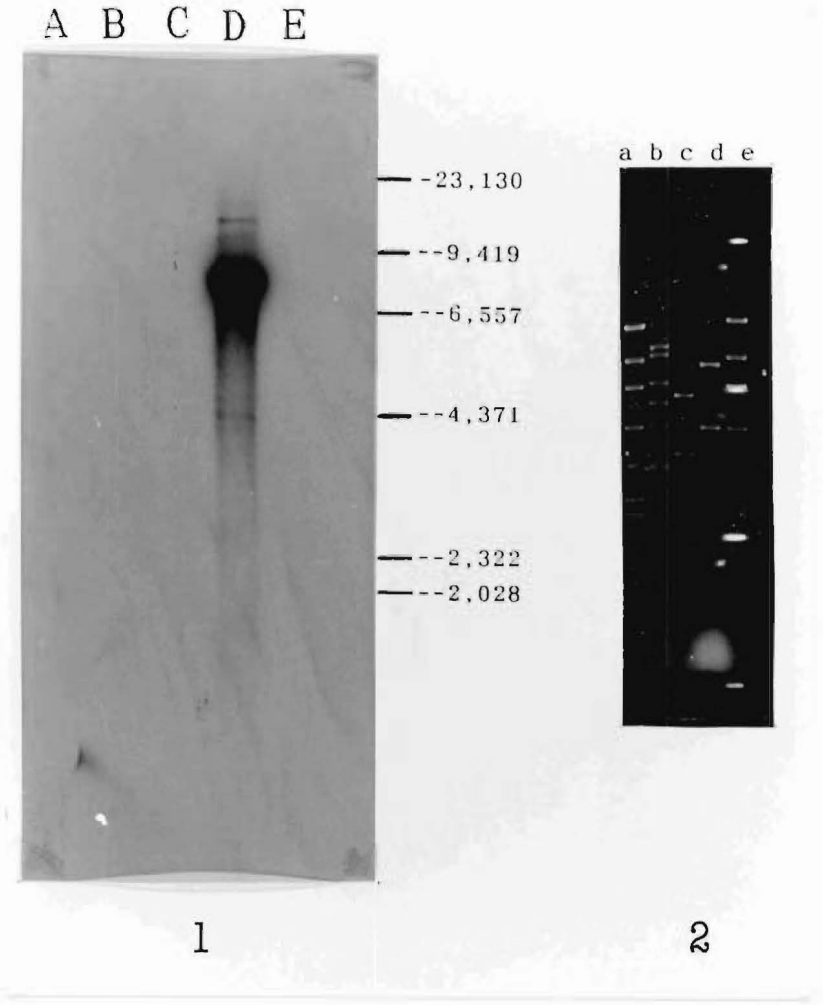


Fig. 4.10. Southern blot analysis of *amb1* and *amb2* locus. The plasmid pSER201A containing the *amb1* locus was digested with *Bam*HI. The 9 Kb insert fragment was isolated to prepare a radioactive probe. 1) Autoradiography of the Southern blot hybridized to ^{32}P labelled probe of the *amb1* locus DNA. Capital letters correspond to the same lanes shown in the gel. 2) 0.7% agarose gel used for the Southern blot. Lanes: a) Lambda/*Hind*III; b) ϕCW1 (marker); c) pSER4/*Hind*III; d) pSER201A/*Bam*HI; e) Lambda/*Hind*III.

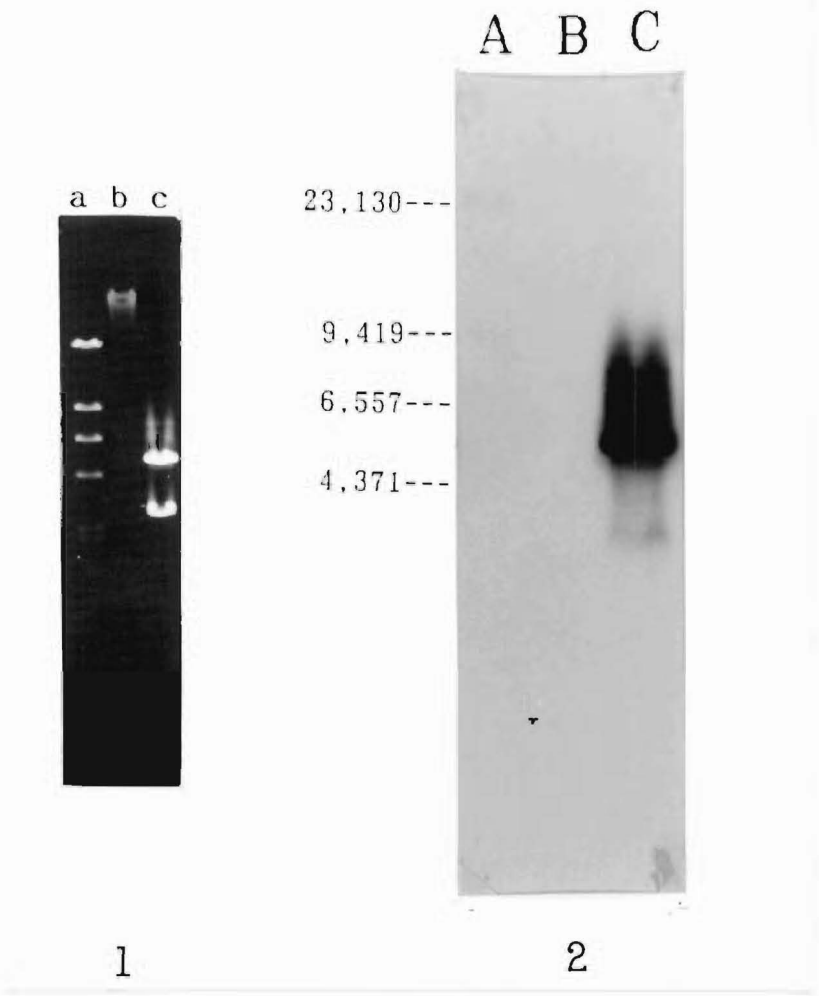


Fig. 4.11. Southern blot analysis of *S. entomophila* plasmid DNA and *amb2* locus. The plasmid pENV2 was digested with *Bam*HI to isolate the 5.3 Kb DNA fragment to prepare a ^{32}P labelled probe. 1) 0.4% agarose gel used for Southern blot. Electrophoresis was carried out overnight at 20 volts. Lanes: a) Lambda/*Hind*III; b) Plasmid isolated from *S. entomophila* UC9 (Path$^+$) ; c) pENV2/*Bam*HI. 2) Autoradiography of Southern blot hybridized to a radioactive probe of the *amb2* DNA. Capital letters correspond to the same lanes in the gel.

Consequently, the *amb2* toxin associated activity seems to be encoded in the bacterial chromosome.

3.4.4) Identification of anti-feeding activity in supernatants of *S. entomophila* broth cultures

An important criterion for judging whether the symptoms of a disease caused by bacteria are the result of a toxin activity, is the ability of such a component to mimic symptoms separately from the bacteria (Van Heyningen, 1955; Rutter, 1988). To date, no component with this feature has been identified for amber disease, but the *amb2* AFE is associated with a toxin-like activity. Then, bioassays of the *S. entomophila* culture filtrates were performed to search for AFE. Pathogenic (UC9) and nonpathogenic (UC7 and UC24) strains were grown overnight for this experiment. Supernatants were filter sterilized and further dialysed to eliminate salts. After dialysis, the filtrates were sterilized again and concentrated three hundred times. Luria broth were equally treated to be used as a control. Samples from the different concentrated supernatants (CS) were tested for bacterial contamination. Pieces of carrot were coated with the CSs to feed the larvae. Results of these experiments are shown in Fig. 4.12. Larvae fed with CS from UC9 showed strong AFE at short period after the inoculation (75.8 %, at day 5; $P<0.001$). Additionally, development of amber color was noticed in this larvae group, though not so acute (48 %, at day 10 after inoculation; $P<0.05$). Conversely, these symptoms were not observed for larvae fed on CS from the nonpathogenic isolates UC7 and UC24. A significant rate of AFE was also observed in larvae fed on LB (45 %; $P<0.05$). However, differences between the AFE that was LB related and AFE that was from the CS from UC9 are significant ($P<0.05$). The larvae fed on LB did not show development of amber color, as was observed with UC9.

3.5) Bioassays with *E. coli* carrying *amb2* locus

The information and results collected during the research suggested that *amb2* locus encoded structural proteins associated with a toxin-like activity. An important test for further research on the *amb2* locus, was to prove the ability of the cloned DNA to be expressed in a *S. entomophila* independent system, such as *E. coli*. This would be a powerful and extra argument supporting the hypothesis that *amb2* locus actually encoded structural proteins with anti-feedant activity. Therefore, bioassays with insect larvae were performed with *E. coli* isolates carrying *amb2* locus.

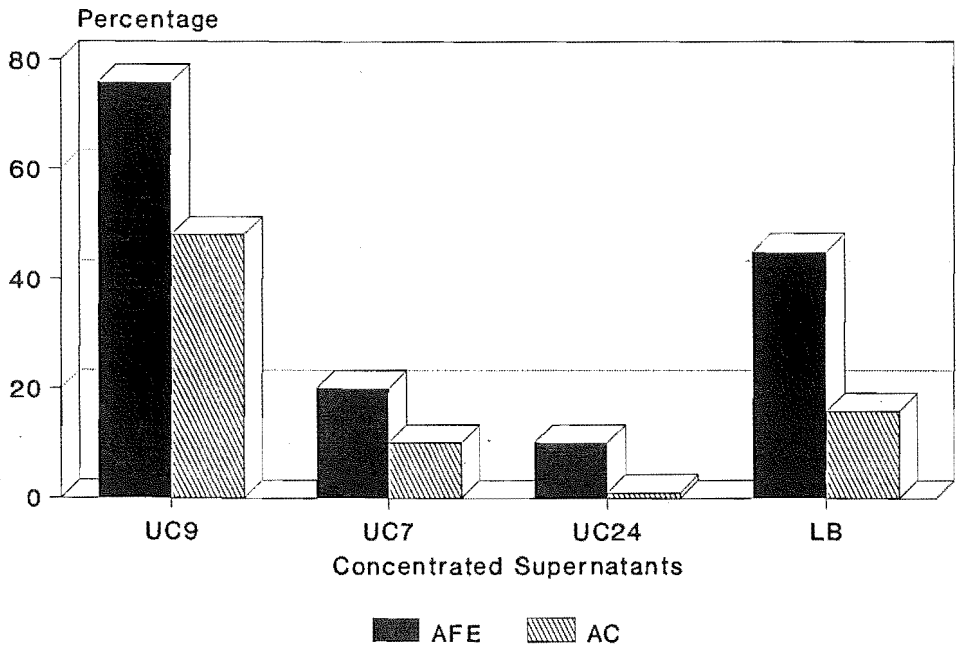


Fig. 4.12. Insect larvae bioassay of culture supernatants of pathogenic and nonpathogenic *S. entomophila* strains. Culture supernatants from UC9, UC24 and UC7 were tested for ability to cause amber disease symptoms. Luria-Bertani broth (LB) was used as control. Anti-feeding effect (AFE) and amber coloration (AC) are in percentage of larvae showing these amber disease symptoms. Percentages are shown after 5 days of inoculation for AFE and after 10 days for AC.

First attempts were made with *E. coli* HB101 bearing *amb2* in the vector pLAFR3 (pENV1A). No effect was observed in the larvae fed with this isolate (Fig. 4.13; $P<0.05$). Since pLAFR3 is a large plasmid (22 Kb) and generates only 3 or 4 copies per cell, *amb2* locus was cloned into the multicopy plasmid pUC18 expecting an increment of *amb2* copies and consequently increments also in the expression levels of AFE.

Cloning was performed by ligation of *Bam*HI digested pENV1 (carrying *amb2* in pLAFR3) and CIP treated pUC18. *E. coli* DH5α was transformed with the ligation mixture and white colonies growing on LB plates supplemented with Ap and X-gal were selected for. Recombinant pUC18 plasmids were confirmed by restriction analysis. The plasmid was named pENV2.

Plasmid pENV2 was transferred to *E. coli* HB101 and the resulting strain was fed to the larvae in the bioassay. Also, HB101 carrying a vector plasmid and the pathogenic *S. entomophila* UC9 were used as controls. Results of these experiments are shown in Fig. 4.13. The highest net AFE rate detected in larvae fed with HB101 bearing *amb2* locus was only 28.8 % ($P<0.05$). No significant differences of amber coloration were observed between this group of larvae and those fed with the control HB101. High levels of AFE were noticed in this control group of larvae (40 %), suggesting that *E. coli* itself might cause some cessation of feeding.

Since a significant difference of AFE was observed between *amb2* locus carried in pLAFR3 and pUC18 (33.3 %; $P<0.05$), a possible dose-dependent effect was suggested. Therefore, a multiple-dose experiment was performed to analyze this possibility. Larvae were fed several times (day 1, 4, 8, 12, 16 and 20) with carrot coated with pathogenic *S. entomophila* (A1MO2), *E. coli* HB101 carrying pENV2 and the same strain bearing only the vector pUC18 as a control. Results are summarized in Fig. 4.14. Amber disease symptoms were marked in larvae fed with A1MO2. No amber color was noticed in both groups of larvae fed with *E. coli*. Increasing rates of AFE were observed in the group fed on *E. coli* carrying pENV2, reaching similar rates to those caused by the pathogenic strain at the end of the bioassay ($P<0.05$). The results sustain the hypothesis of a toxin-like protein encoded by *amb2* locus and also, that the AFE is dependent on the dose of such protein consumed by the larvae.

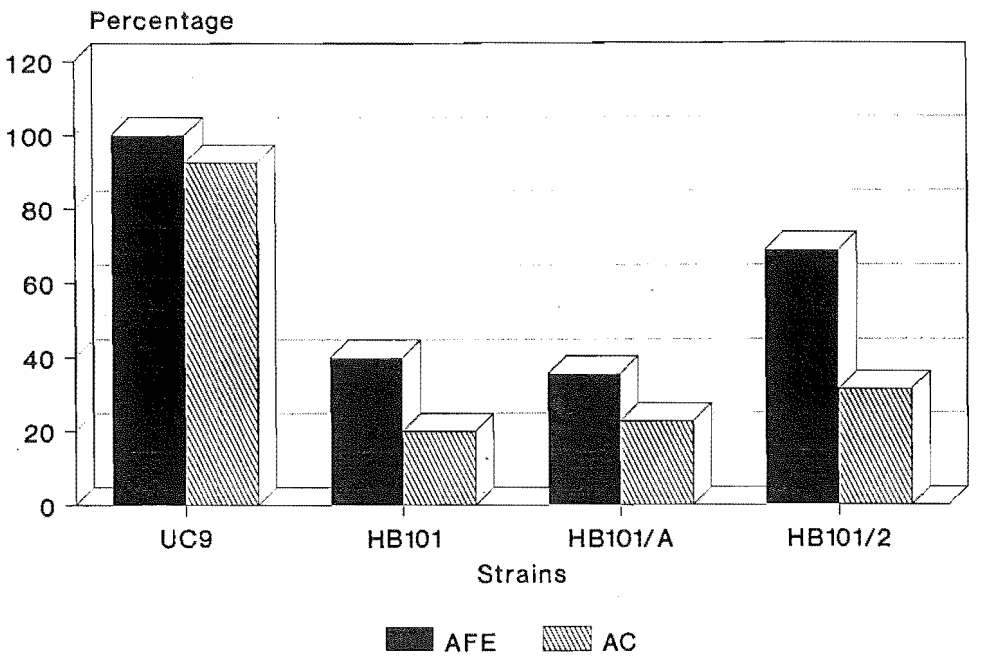


Fig. 4.13. Insect larvae bioassay of E. coli HB101 carrying *amb2* locus. Plasmids pENV1 OriA and pENV2 containing *amb2* locus, were introduced into *E. coli* HB101 and tested for ability to cause amber disease symptoms (HB101/A and HB101/2, respectively). The positive control was UC9. HB101 carrying pLAFR3 was used as negative control. Anti-feeding effect (AFE) and amber coloration (AC) are in percentage of larvae showing these amber disease symptoms. Results based on 40 larvae per treatment after 13 days of inoculation.

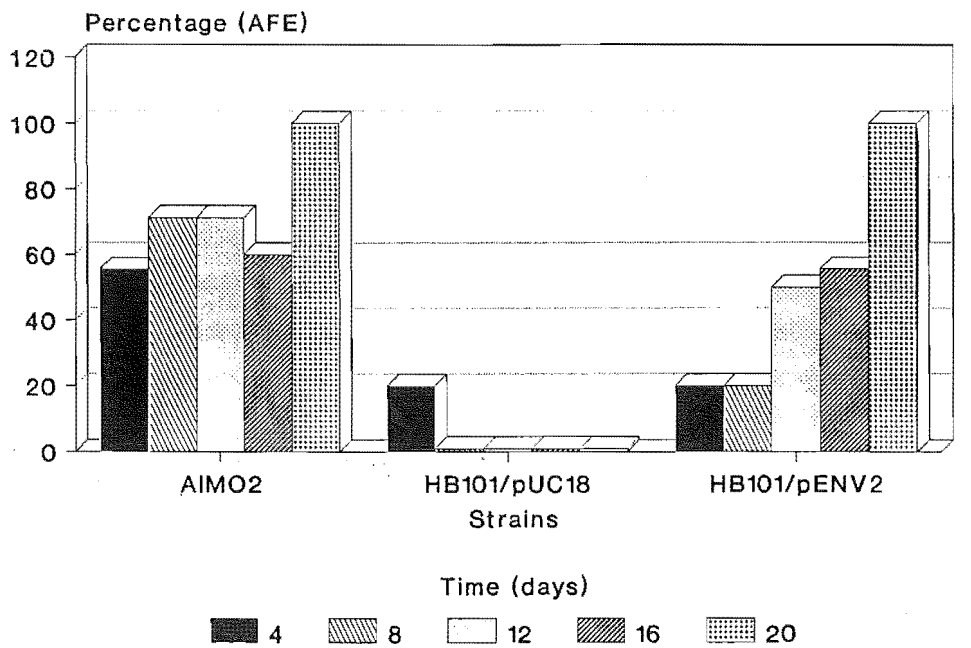


Fig.4.14. Insect larvae bioassay with multiple-dose of *E. coli* HB101 carrying *amb2* locus. Plasmid pENV2 containing *amb2* locus, was transferred to *E. coli* HB101 and tested for ability to cause anti-feeding effect. The positive control was A1MO2. HB101 carrying pUC18 was used as negative control. Five doses of bacteria were used to feed the larvae, each one at 4 days intervals. Percentages of larvae showing anti-feeding effect (AFE) is presented. Results based on 10 larvae per treatment.

4) DISCUSSION

Looking for the identification of *S. entomophila* DNA sequences encoding for pathogenic determinants of amber disease to *C. zealandica*, a 5.3 Kb genetic region was isolated conferring anti-feeding effect to the scarab larvae. The genetic locus has been designated *amb2* as it is the second locus identified associated with the disease.

Six cosmids carrying wild type DNA sequences where the Tn*phoA* insertion site in UC24 maps, were isolated from the wild type *S. entomophila* library. By physical mapping, a 5.3 Kb DNA region containing the *amb2* locus was identified. The UC24 mutant locus maps in *amb2*. This DNA fragment was isolated and cloned to create pENV1.

Complementation experiments were performed in UC24 with pCOS31, pCOS35 and pENV1 containing *amb2* locus. Controls on the stability of the plasmids inside the bacteria were important since *S. entomophila* is a wild type strain and lacks a recombination deficient phenotype (Rec-). The results showed a partial complementation of the nonpathogenic mutant UC24 with DNA sequences contained in the recombinant cosmids. This DNA fully restored the anti-feeding effect (AFE) in UC24 and partially restored the ability of the mutant to cause development of amber coloration (AC). Thus, *amb2* seems to be related with both the anti-feeding effect and the development of amber coloration. However, its effect in the former apparently is stronger than in the latter. The results suggested the expression of functional protein(s) by the DNA sequences of *S. entomophila* present in the cosmids. These protein(s) may be directly associated with the AFE and indirectly with amber coloration, since the effect on this symptom was not strong. It was expected a full restoration of amber disease symptoms with the recombinant cosmids in UC24. The observed deficiencies in ability to cause amber coloration might be explained by competition between functional proteins encoded by the cosmid and deficient proteins expressed by UC24 mutant genes.

The rates of AFE and AC were similar when UC24 was carrying either, pCOS31, pCOS35 or pENV1. This fact strongly suggested ($P<0.001$) that the locus causing cessation of feeding in the grass grub larvae was contained in the 5.3 Kb DNA fragment. However, it was questioned whether the Anf^+ phenotype (ability to cause AFE) restored in UC24, was the result of two possible events: i) a toxin-like

protein activity, directly causing AFE; or ii) it was the result of proteins with a regulatory activity controlling a virulence regulon, therefore restoring amber disease symptoms.

Three factors were considered in order to clarify the function of *amb2*: i) pathogenicity in *Enterobacteriaceae* is controlled by a very complex and multifactorial mechanism ; ii) pathogenic determinants may work individually or combined to produce infection and disease, and iii) pathogenic organisms use regulatory cascades to control the expression of virulence genes (Finlay and Falkow, 1989; Mims, 1987; Finlay, 1992; Dorman and Bhriain, 1992; DiRita, 1992; DiRita, *et al.*, 1992). Thus the effect of *amb2* on the phenotype of several strains of *S. entomophila* was analyzed. Four phenotypes were pondered: pathogenicity, MRE-HA fimbriae, protease and chitinase activity.

The nonpathogenic mutants UC21, UC7 and UC57 were used for testing restoration of Anf^+ phenotype by *amb2*. Pathogenicity tests showed that AFE was fully restored in UC7 carrying *amb2*. Low levels of AFE were observed in UC21 and UC57 carrying *amb2*. Even though the AFE rate was not statistically significant in UC57 ($P>0.05$), the results suggest that the Anf^+ phenotype is partially restored in both mutants by *amb2*. However, it is evident that *amb2* alone is not sufficient for maximal expression of AFE in these mutants.

The different expression levels of *amb2* in the three strains might be explained by the different nature of the mutants. UC21 has a Tn*phoA* inserted into the *amb1* locus associated with the inhibition of pili expression. This fact seems to produce deficiencies in the ability of the bacteria for adhering to the larvae gut (Upadhyaya, *et al.*, 1992). These experiments showed also that the mutant UC21 is unable of causing cessation of feeding. This observation suggested that adhesion of the bacteria to the larvae gut was essential for the appearance of amber disease symptoms. Further bioassays showed increasing expression of AFE displayed by larvae fed on UC21 during the bioassay (results not shown). As UC21 has a mutation only in *amb1* locus is reasonable to expect expression of Anf^+ phenotype while its genetic locus is unaltered. Therefore, it appears that maximal levels of AFE caused by *amb2* requires efficient expression of *amb1* locus.

Results shown in Chapter III suggested that the Tn*phoA* insertion in UC57 was related with a regulatory gene(s). High AFE levels similar to those showed by

the wild type strain UC9, apparently require a full virulence network unaltered, so *amb2* expressed in UC21 and UC57 cannot reach those levels. On the other hand, UC7 is a spontaneous nonpathogenic mutant. The nature of the mutation is unknown, although previous information suggested alterations on its ability for adhering to the larvae gut (Wilson, 1988). However, UC7 seems to have the *amb1* locus unaltered (Upadhyaya, *et al.*, 1992) and no differences were found on its ability to agglutinate erythrocytes as compared with the haemagglutination pattern of the wild type *S. entomophila* (see Chapter II), suggesting no changes on its adhesion abilities to the larvae gut. On the contrary, UC21 showed deficiencies on the MRE-HA, phenomenon that has been associated with deficiencies in pili and/or adhesin expression (see Chapter II). The higher rates of AFE and besides, the ability to develop amber coloration observed in UC7 carrying *amb2*, suggest also the presence and activity in this strain of positive regulatory genes controlling *amb2* locus and/or the association with other virulence determinants (i.e., pili) to produce amber disease symptoms.

The nonpathogenic strains UC21 and UC57 are Tn*phoA* mutants and UC7 is a spontaneous Km^r, nonpathogenic mutant. The three strains are derivatives of the $Path^+$ strain UC9 and therefore contain the genetic potentialities to cause amber disease. This potentialities involve the regulatory network to control virulence genes. Then, this network might control, in turn, the *amb2* locus. However, it became evident that their respective mutations did not allow the full restoration of amber disease symptoms. These facts could explain the *amb2* activity (AFE) expressed by those three strains compared with no activity at all in A20. Since this strain is a naturally occurring nonpath isolate, it lacks probably the pathogenicity genetic network and *amb2* locus is unable to express efficiently in this condition. Other possibility is that A20 may have a point mutation in an essential gene.

The AFE caused by pENV1 in UC7 was stronger than the AFE caused by the library cosmids pCOS31 and pCOS35 which contain 30 and 24 Kb DNA inserts. This fact suggest that some regulatory sequences adjacent to the 5.3 DNA region might regulate, at least in part, the *amb2* locus. However, the increment of anti-feeding effect displayed by pENV1, could be also explained by faster replication and transcription of *amb2* related with the smaller plasmid size.

Prior information suggested that the genetic locus altered in the nonpathogenic mutant UC24 (*amb2* locus) was involved in piliation (Upadhyaya, *et*

al., 1992). However, as mentioned previously, no differences in the MRE-HA pattern were observed among the following strains: i) the pathogenic *S. entomophila* UC9 and A1MO2; ii) the nonpathogenic mutants UC24 and UC7 ; and iii) these mutants carrying *amb2* locus sequences (Table 4.4). Since the MRE-HA fimbriae have been related with pathogenicity (see Chapter II), then, the locus amb2 apparently is not associated neither with fimbriae nor adhesion of the bacteria to the larvae gut. Supporting these results, is the fact that no homology was detected by Southern analysis between *amb2* and *amb1* locus, which was associated with fimbriae production and adhesion of the pathogen to the insect gut (Upadhyaya, *et al.*, 1992).

Because no relationship was found either between the *amb2* locus and the expression of proteases and chitinases (Table 4.4), it is proposed that the Anf^+ phenotype displayed by *amb2* is not the result of a regulator activity and is caused by a different factor not associated with MRE-fimbriae, proteases or chitinases.

Some expression of Anf^+ phenotype was observed in *E. coli* carrying the *amb2* locus cloned in the multicopy plasmid pUC18 (pENV2). However, this expression level was just 39% of that showed by *amb2* in UC24. A multiple dose-experiment showed that pENV2 caused increasing AFE rates during the bioassay, reaching percentages similar to those produced by the pathogenic strain A1MO2. Even though the expression of *amb2* in *E. coli* seems to be relatively low compared with that in *S. entomophila*, these results provide strong evidence that *amb2* contains DNA sequences encoding proteins with a toxic activity against larvae of *C. zealandica.*

Very likely, some regulatory proteins encoded by the *Serratia* genome are important for an efficient expression of *amb2*. Some proteins with toxic properties need to be processed to be active (Middlebrook and Dorland, 1984; Höfte and Whiteley, 1989) and *Serratia* proteases could play an important role in this activation. Lack of these factors in *E. coli* may cause lower anti-feeding effect by *amb2* sequences than that showed by *Serratia* strains. Pearson and Mekalanos (1982) noted several observations about the expression of cholera toxin in *E. coli*: i) the amount of toxin produced in *E. coli* was about 100 fold less than that produced by *V. cholerae*; ii) a 200-fold reduction in the specific activity of the toxin (in terms of tissue culture toxicity versus toxin antigen), due to the lack of proper proteolytic processing (nicking); and iii) the cholera toxin produced by *E. coli* was found to be cell associated rather than extracellularly secreted as it is when produced by *V. cholerae*. The low expression of *amb2* in *E. coli* might share some features like those

of cholera toxin.

Development of amber coloration was not observed in larvae fed with *E. coli* carrying *amb2* locus. This result might be explained by a dose-related effect and/or the lack of other important factors acting as a complement or "enhancers" of the toxin activity in *S. entomophila.* Such factors could be some enzymes as proteases deteriorating the immune insect response as it has been shown in other systems (Madziara, *et al.*, 1971; Kaska, 1976; Flyg and Xanthopoulos, 1983) and/or chitinases probably involved in the degradation of the larvae gut (Glare and Jackson, 1990; Glare, *et al.*, 1992) causing general stress to the larvae and opening a pathway for later invasion of the insect hemocoel. The adherence phenomenon could be important also for an action of the different factors involved, directly onto the cells of the gut.

Amber disease symptoms were observed in larvae fed with concentrated supernatants (CS) of cultures from the Path$^+$ strain UC9. Larvae fed with CS from the nonpathogenic strains UC24 and UC7 did not show any amber disease symptom. The results strongly suggest that *S. entomophila* UC9 secretes toxins which are responsible for amber disease symptoms and support previous hypothesis on the involvement of a toxin in this disease (see Chapter 1). The low level of AC observed in larvae fed with CS from UC9 might be associated with two factors: i) with a dose-dependent activity of the toxic factors present in the supernatants; or ii) with the requirement of other virulence factors essential to accomplish a full toxic activity. In fact, the supernatants were 300 times concentrated and, on the other hand, it has been shown that AFE caused by *amb2* is not fully restored in *S. entomophila* mutants deficient in virulence factors (e. g. UC21 and UC57).

A significant level of AFE was also observed in the control larvae fed with LB. This effect resembles similar AFE values observed in larvae fed only with carrot (for comparisons see Fig. 4.5, 4.7, 4.8 and 4.9). The rates are higher than those showed by larvae fed with nonpathogenic larvae. These facts suggest: i) that bacterial populations living in the larvae gut in small numbers, either pathogenic *S. entomophila* or other species may cause larvae cessation of feeding when nutrients are available (i.e. carrot or LB) and growth of the microbial population is favoured; ii) that nonpathogenic *S. entomophila* might compete in the larvae gut against other inhabitants and possibly produce bacteriocins. The production of this type of proteins active against *E. coli* and other bacterial species has been reported in *S.*

marcescens (Traub, 1980). The effect of bacteriocins on other bacterial inhabitants might decrease the AFE levels caused by them. This hypothesis might explain lower levels of AFE observed with CS from nonpath strains, compared with AFE observed in larvae fed with LB and carrot. Accordingly, AFE rates resulting from UC9 supernatants should be compared with AFE values of CS from nonpath strains and not with LB rates.

Southern analysis of the *amb2* locus and the plasmid from *S. entomophila* UC9 showed no hybridization between both DNAs. This result strongly suggests that the *amb2* locus is located in the bacterial chromosome. Some toxins are plasmid born, as the enterotoxins produced by *E. coli* (Elwell and Shipley, 1980) whereas others are integrated into the bacterial genome, as the *ctx* genes encoding cholera toxin (Vasil, *et al.*, 1975). Therefore, it is not rare that a the *amb2* toxin-like protein is encoded by *S. entomophila* chromosomal genes.

Upadhyaya, *et al.*, (1992) reported unstability in the Path$^-$ phenotype of *S. entomophila* UC24. However, no alteration was observed in the UC24 Path$^-$ phenotype in this work. The unstability previously reported might be explained by the nature of the mutant, where the transposon may move and reinsert into another region of the *S. entomophila* genome allowing the reestablishment of the Path$^+$ phenotype in some derivatives of UC24.

In summary, I have identified, isolated and cloned a *S. entomophila* genetic locus, amber disease associated rendering anti-feeding effect to grass grub larvae. The genetic region has been called *amb2*. Genetic evidence has been presented suggesting that *amb2* encodes an anti-feedant toxin responsible of AFE. Further evidence showing that the *amb2* gene(s) products actually cause AFE is required. The following chapter describes the molecular characterization of the cloned genes in *E. coli* for finding out the *amb2* encoded proteins, the genes structure and their regulation. These approaches will be useful for elucidating the nature of the anti-feedant toxin and the basis of its action, as well as its specificity.

CHAPTER V

MOLECULAR CHARACTERIZATION OF *amb2* LOCUS

1) INTRODUCTION

A *S. entomophila* 5.3 Kb DNA fragment associated with amber disease has been isolated and cloned. This fragment has been designated *amb2* and contains a locus conferring anti-feeding effect on larvae of *C. zealandica.* In order to identify the *amb2* gene products a minicell protein analysis system was used (Adler, *et al.*, 1967). The main advantage of this system is that these special cells express exclusively the proteins encoded by a plasmid. Some strains of *E. coli* as P678-54T, divide asymmetrically producing small anucleated cells. When the parental bacteria are carrying a plasmid, the produced minicells contain at least one molecule of that plasmid. By cloning the *amb2* DNA fragment into pBR322 and analyzing the expression products of the minicells carrying the recombinant plasmid, two proteins were identified: AnfA and AnfB. These proteins seem to be responsible for the anti-feeding effect.

2) MATERIALS AND METHODS

2.1) Bacterial strains and vectors

The bacterial strains and vectors for cloning utilized in this work are described in Table 5.1 and 5.2. Media and growth conditions are described in Chapter II, section 2.1.

2.2) DNA manipulations, restriction analysis and cloning

General methods were performed as previously described (see Chapter III, section 2.6). Electroporation was used to transfer plasmids to *S. entomophila* strains and *E. coli*, when conventional methods for transformation were not efficient (see Chapter III, section 2.7).

2.3) Hybridization analysis

Procedures for Southern blot analysis and preparation of radioactive probes

Table 5.1. Bacterial strains used in this study

Strain	Relevant Characteristics	Reference
S. entomophila		
A1MO2	Wild-type; Path$^+$	T.A. Jackson
UC9	Clonal selection from A1MO2;Ap^r Path$^+$	H.K. Mahanty
UC7	Spontaneous Km^r mutant from A1MO2; Ap^r Km^r Path$^-$	H.K. Mahanty
UC24	Tn*phoA* mutant derivative from UC9; Path$^-$ Ap^r Km^r Gm^r	195
E. coli		
HB101	F$^-$ *pro leu thi lacY recA hsdR hsdM*	166
DH5α	F$^-$ *lacZ* M15 *endA1 hsdR17 supE44 thi-1 gyrA relA1*	171
P678-54T	*thr-1 leu-6 thi-1 lacy1 xyl-7 ara-13 mtl-2 gal-6 rpsl tonA2 minA minB pil*	4
POII1681	M8820 with Mu dII1681 *ara*::(Mu *cts*)3 *araD*$^-$ *leu*$^+$ *lac*$^+$ *pro*$^-$	31
MH3497	recA$^+$ *lac gal rps* L Mu *cts* 62	Lab stock

Table 5.2. Plasmids used in this study

Plasmid	Relevant Characteristics	Reference
pBR322	ColE1 *ori* Ap^r Tc^r; cloning vector; 4.36 Kb	15
pUC18	ColE1 *ori lacZ* Ap^r; cloning vector; 2.69 Kb	200
pLAFR3	pRK290 derivative with lambda *cos lacZ* and polycloning sites of pUC9; RP4 (IncP-1) *ori* Tc^r; cloning vector; 22 Kb	184
pBLUESCRIP	phagemid cloning vector, KS; Ap^r *lacZ*; 2.95 Kb	Stratagen
pSER4	9 Kb *Bam*HI insert containing part of Tn*phoA* and flanking *S. entomophila* DNA from UC24 cloned into pBR322; Ap^r Km^r	H.K. Mahanty
pENV1	*Bam*HI insert fragment of 5.3 Kb containing *amb2* from pCOS31 cloned in pLAFR3; Tc^r	This work
pENV2	*Bam*HI insert fragment of 5.3 Kb containing *amb2* from pENV1 cloned in pUC18; Ap^r	This work
pANF4A	*Bam*HI insert fragment of 5.3 Kb containing *amb2* from pENV2 cloned in pBR322; Ap^r	This work
pANF4B	Same as pANF4A but with the 5.3 Kb DNA insert in opposite orientation	This work
pANF22	pANF4A with a 3.1 Kb *Hind*III fragment of *amb2* deleted This plasmid contains the *anfB* gene; Ap^r	This work
pANF135	pANF4A with a 3.4 Kb *Hind*III fragment of *amb2* deleted This plasmid contains the *anfA* gene; Ap^r	This work
pANF13	pANF4A with a 3.9 Kb *Hind*III fragment of *amb2* deleted This plasmid contains part of the *anfA* gene; Ap^r	This work
pENV22	*Hind*III 2.1 Kb insert fragment of pANF22 recloned into the same site of pLAFR3; Tc^r	This work
pENV135	*Eco*RI-*Bam*HI 1.9 Kb insert fragment of pANF135 recloned into the same sites of pLAFR3; Tc^r	This work
pENV13	*Eco*RI-*Bam*HI 1.4 Kb insert fragment of pANF13 recloned into the same sites of pLAFR3; Tc^r	This work
pMMU5	pANF4A with a Mu-*lac* insertion in the *anfB* gene of *amb2* locus; Ap^r Km^r lac^+	This work
pMMU24	Same as pMMU5 but with the insertion point at the right side of that one; Ap^r Km^r lac^+	This work
pMMU36	pANF4A with a Mu-*lac* insertion in the *anfA* gene of *amb2* locus. Insertion into the 375 bases *Hind*III DNA fragment; Ap^r Km^r lac^+	This work
pMMU37	pANF4A with a Mu-*lac* insertion at the left side of that in pMMU5; Ap^r Km^r lac^+	This work
pMMU48	pANF4A with a Mu-*lac* insertion in the *anfA* gene of *amb2* locus. Insertion at the right of that one in pMMU36; Ap^r Km^r lac^+	This work
pENV11	*Eco*RI-Kpn1 DNA insert of 1.1 Kb from pANF13 cloned into the same sites of pBLUESCRIPT KS; Ap^r	This work
PENV16	*Eco*RI-*Kpn*I DNA insert of 1.6 Kb from pANF16 cloned into the same sites of pBLUESCRIPT KS; Ap^r	This work
pENV05	*Hind*III insert fragment of 0.5 Kb from pANF135 cloned into the same site of pBLUESCRIPT KS; OriB; Ap^r	This work
pENV03	*Hind*III insert fragment of 0.3 Kb from pANF135 cloned into the same site of pBLUESCRIPT KS; OriA; Ap^r	This work
pENV10	*Hind*III insert frgament of 1 Kb from pANF135 cloned into the same site of pBLUESCRIPT KS; OriB; Ap^r	This work

are described in Chapter IV, section 2.3.

2.4) Minicell analysis

The *E. coli* minicell strain P678-54T was used to determine the gene products of recombinant plasmids. After transformation by electroporation, minicells were grown overnight on plates with suitable antibiotics. Several loopfuls of cells from the plate were resuspended in 1 ml of LB to an OD_{600} of ~0.8. Five hundred µl were added to a 500 ml Erlenmeyer flask containing 150 ml of LB (2 flasks for each strain). The cells were vigorously shaken overnight to an OD_{600} of ~0.8-1. The cultures were centrifuged at 2K, 7 min at 15°C (Sorvall, rotor GSA). The pellets were discarded and the supernatants containing minicells were centrifuged at 10K, 10 min at 15°C (Sorvall, rotor GSA). The pellets from 150 ml of culture, were dissolved in 2 ml of BSG buffer (8.5 g NaCl/l, 0.15 g KH_2PO_4/l , 0.30 g Na_2HPO_4/l, 50 mg gelatin/l). The cell suspension was placed onto a 35 ml sucrose gradient in a 50 ml clear centrifuge tube. The tubes were centrifuged at 5 K, 4°C, 15 min, in a swinging rotor (Heraeus HB-4 rotor or Beckman SW27 rotor). Ten ml from the upper minicells band were carefully removed and transferred to a 25 ml centrifuge tube containing 5 ml of BSG buffer. The minicells were pelleted by centrifugation at 10 K, 10 min, 15°C (Sorvall SS34 rotor). The pellet was redissolved in 2 ml of BSG buffer and loaded onto a second sucrose gradient. The minicells pellet from the second gradient was resuspended in 10 ml of BSG buffer to measure the OD_{600}. The cells were again pelleted (10 K, 10 min, 15°C, Sorvall SS34 rotor) and resuspended in minimal medium (M63) containing 30% glycerol to an OD_{600} of 2. Ten µl of this suspension were plated out to assess the level of viable cells contamination; up to 10^2 viable cells on the plate did not interfere with the detection of minicell-directed polypeptide synthesis. Minicells were stored in aliquots of 100 µl at -20°C until further use.

2.4.1) Preparation of sucrose gradients

Step gradients were prepared in 50 ml clear centrifuge tubes by layering (from bottom to top) 12 ml of 20%, 12.5% and 5% sucrose solution (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.1 mM EDTA, 20%, 12.5%, 5% sucrose).

2.4.2) Labelling proteins of minicells

Frozen minicells (aliquots of 100 µl) were placed on ice 10 min and adjusted

to OD_{600} 0.2 by adding 900 µl of the following labelling buffer:

2X Minimal medium (M63)	5.0 ml
100 mM $MgSO_4$	0.2 ml
20% glucose	0.2 ml
1% B_1 (thiamine)	0.040 ml
100X aminoacids mixture (with no methionine)	1.9 ml
H_2O	2.66 ml
Total volume	10.00 ml

The minicells were incubated at 37°C with gentle agitation. After 20 min, ^{35}S-methionine (20 µCi/ml) was added. Cells were placed again at 37°C with gentle agitation during 30 min for labelling and further pelleted in microcentrifuge tubes. The samples were resuspended in 60 µl of B buffer (7 g Na_2HPO_4/l, 3 g K_2HPO_4/l, 4 g NaCl/l, 0.1 g $MgSO_{4/l}$). Sixty µl of 2X treatment buffer (0.125 M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol) were added for SDS-PAGE. Samples were boiled 3 min and loaded onto the gel. Following electrophoresis, radioactive polypeptides were detected by autoradiography.

2.5) SDS-PAGE

Proteins from minicells were analyzed by SDS-PAGE prepared by conventional procedures basically as reported (Laemmli, 1970).

2.6) Mini-Mu mutagenesis

Plasmids were introduced to *E. coli* strain POII1681 by transformation or electroporation. Bacteria carrying plasmids were grown overnight at 30°C in SB (super broth) containing suitable antibiotics. Two hundred µl of this culture were transferred into a flask containing 10 ml of LB with antibiotics. The cultures were incubated at 30°C, shaking for 4½ hrs. At this temperature, the Mu repressor gene *c* is active. The cells were transferred to 45°C for 30 min. and then at 37°C with strong agitation. Under these conditions the repressor is inactivated and phage lytic growth is favoured. Total lysis occurred after 100 min. Chloroform was added (30 µl), the sample was mixed by vortex and incubated 5 min RT. An aliquot of 1.5 ml of the lysate was transferred to a microcentrifuge tube, 3 drops of chloroform were added

and the tube was vortexed and centrifuged 1 min. One ml from the top was transferred to a new tube to be used for transduction.

E. coli strain MH 3497 was grown overnight at 30°C. One hundred µl of the culture were placed in a centrifuge tube containing 5µl of 100 mM $MgSO_4$ and 2.5 µl of 100 mM $CaCl_2$. After the addition of 10 µl of the Mu lysate, the sample was incubated 20 min at 25°C for absorbtion of the phage. LB was added (1 ml) to proceed incubation at 30°C. The sample was centrifuged (6K, 1 min) and the pellet resuspended in 100 µl of LB to be plated out on agar plates containing antibiotics and X-gal. The plates were incubated overnight at 30°C and the blue colonies were further analyzed.

2.7) DNA Sequencing

Specific restriction fragments were cloned into pBLUESCRIPT (KS M13-) vector and sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977). The $^{T7}Sequencing_{TM}$ Kit (Pharmacia LKB) was used. Double stranded DNA was isolated using standard procedures, treating the samples with LiCl and RNAase to eliminate RNA contamination. Samples were also extracted with phenol-chloroform to remove proteins. DNA concentration was determined by spectrophotometer and the relation OD_{260}/OD_{280} was ~1.7-1.8. Annealing and sequencing reactions were performed according to Pharmacia specifications. The acrylamide concentration of the sequencing gels was 6%. Electrophoresis was performed on a Macrophor system apparatus (Pharmacia LKB) at 1500 V as suggested by the manufacturers. Urea was removed by washing the gel twice with 10% v/v acetic acid during 15 min. The gels were dried 2 hrs at 65°C or at RT overnight and exposed to an X-ray film for autoradiography.

2.8) Bioassays with insect larvae

Procedures of the bioassays for pathogenicity tests are described in Chapter IV, section 2.6.

3) RESULTS

3.1) Determination of the proteins encoded by *amb2* locus

The plasmid pENV2 was *Bam*HI digested and the isolated 5.3 Kb DNA fragment corresponding to *amb2* locus was ligated with dephosphorylated *Bam*HI-digested pBR322. The *E. coli* strain HB101 was transformed with the ligated mixture and recombinants were selected by Ap^r and Tc^s. The insertion was confirmed by agarose gel electrophoresis. Both orientations of the insert were obtained. The recombinant plasmids were named pANF4, OriA and OriB (Fig. 5.1). The *E. coli* strain P678-54T was transformed by electroporation, separately with both plasmids and also with pBR322 to be used as a control. Minicells from the transformants were labelled with [^{35}S] methionine and total cell protein of minicells was analyzed by SDS-PAGE. Results of this analysis is shown in Fig. 5.2. The main proteins expressed by the control pBR322 (Fig. 5.2 and 5.4, lanes A) were those related to ß-lactamase whose unprocessed molecular weight is 32 kDa. A smaller peptide around 29 kDa seems to be the processed form of the enzyme (Labigne-Roussel, *et al.*, 1985). The minicells carrying the plasmid pANF4, OriA expressed at least three proteins (Fig. 5.4, lane B). The molecular sizes were estimated to be 25, 21 and 17 kDa. Analysis of the gene products synthesized from the same plasmid, OriB showed three polypeptides as well (Fig. 5.2, lane B; Fig. 5.4, lane C); a protein of estimated molecular weight of 42 kDa was detected in addition to the 25 and 21 kDa proteins. The 17kDa protein seems to be not expressed when *amb2* locus is in OriB (Fig. 5.2, lane B and 5.4, lane C). Similarly, the 42 kDa product is not present in OriA. Then, *amb2* locus apparently directs only the synthesis of the 25 and 21 kDa proteins. Since the anti-feeding effect has been produced by the plasmid pENV1 carrying *amb2* locus in both orientations (Chapter IV, Fig. 4.5), it is concluded that the 21 and 25 kDa proteins are responsible for that effect. The proteins have been named AnfA and AnfB respectively.

3.2) Deletion mutagenesis

In order to define the DNA regions encoding AnfA and AnfB proteins, three deletion mutants were constructed. Advantage was taken of the multiple *Hind*III restriction sites along the *amb2* DNA region (Fig. 5.3). The plasmid pANF4 containing *amb2* in the 5.3 Kb DNA fragment, was partially digested with *Hind*III and then religated. *E. coli* HB101 was transformed with the ligation mixture and selected for Ap^r. The plasmids from 50 colonies were anclyzed by *Hind*III digestion and agarose gel electrophoresis. Most of the religated plasmids were the original plasmid pANF6. Only three different deletion mutants were isolated by this procedure and were named pANF22, pANF135 and pANF33 (Fig. 5.3). After the

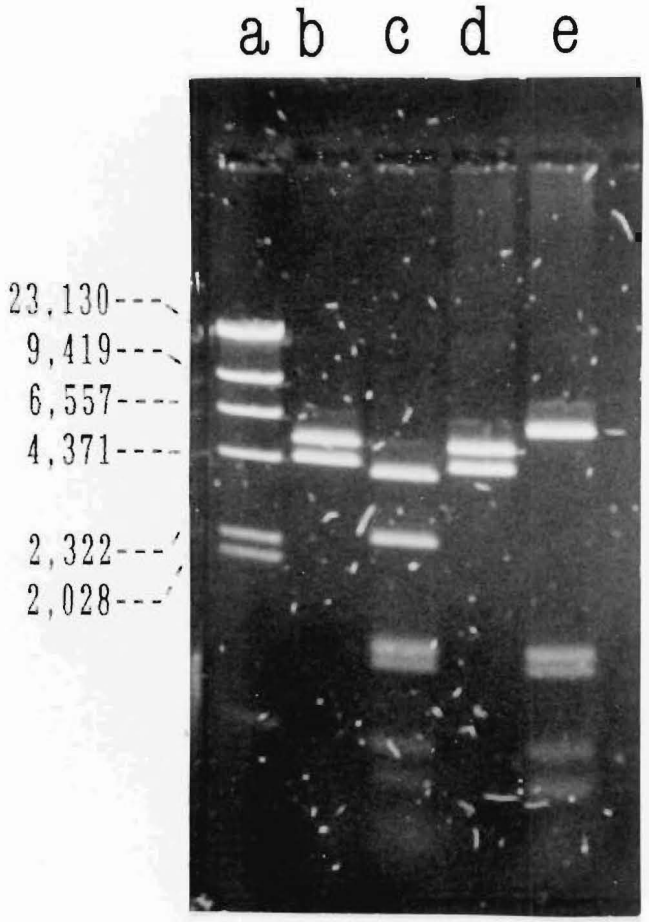


Fig. 5.1. Agarose gel electrophoresis of *amb2* locus cloned in pBR322. The 5.3 Kb DNA fragment isolated from pENV2 was ligated with pBR322 previously digested with *Bam*HI. Both orientations of the insert were obtained and named pANF4 OriA and pANF4 OriB. Lanes: a) Lambda/*Hind*III; b) pANF4 OriA/*Bam*HI; c) pANF4 OriA/*Hind*III; d) pANF4 OriB/*Bam*HI; e) pANF4 OriB/*Hind*III.

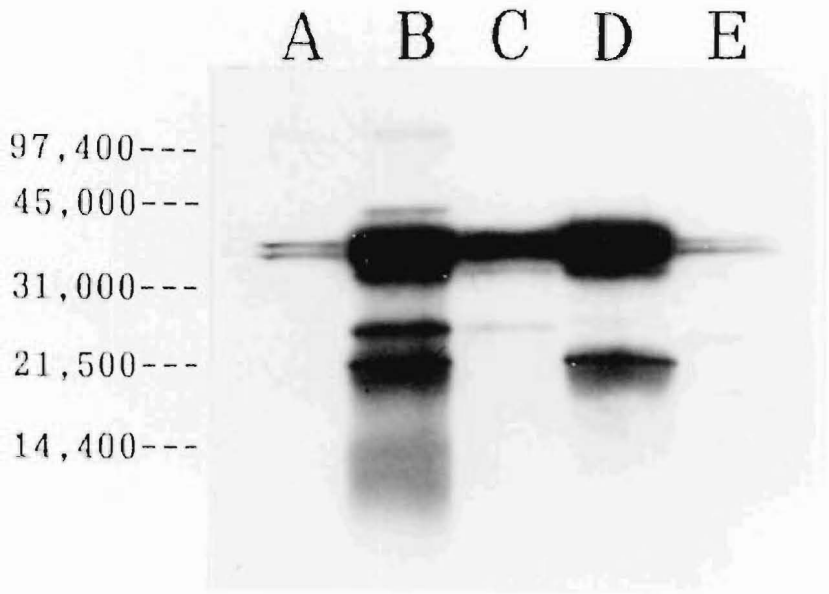
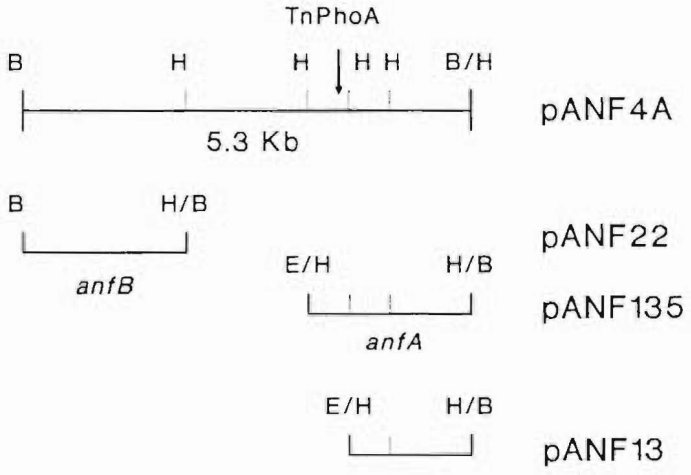


Fig. 5.2. Gene products of *amb2* locus and deletion mutants. SDS-PAGE of plasmid-mediated polypeptides expressed in minicells labelled with [^{35}S]methionine. Lanes: A) pBR322; B) pANF4 OriB; C) pANF22; D) pANF135; E) pANF13. The physical map of these plasmids is in Fig. 5.3. The pBR322 products are shown as a control; molecular sizes of standard proteins are marked in Daltons (Da). Electrophoretic conditions: 15% polyacrylamide gel; the run was at 190 Volts for 45 min at 4°C.

Fig. 5.3. Physical map of recombinant plasmids carrying *amb2* locus and deletion mutants. Part A: The Tn*phoA* insertion site in mutant UC24 is indicated in pANF4A (pANF4 OriA). The subclones containing *anfA* and *anfB* genes in pANF135 and pANF22 are also indicated. Restriction enzymes: E, *Eco*RI; H, *Hind*III, B, *Bam*HI. The gene products of these plasmids expressed in minicells are in Fig. 5.2. Part B) 0.7% agarose gel electrophoresis of *amb2* deletion mutants cloned in pBR322: a) Lambda/*Hind*III; b) pANF22/*Bam*HI; c) pANF13/*Eco*RI-*Bam*HI; d) pANF135/*Eco*RI-*Bam*HI.

A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B
H
H
H H
B/H
5.3 Kb
pANF4A
B
H/B
anfB
pANF22
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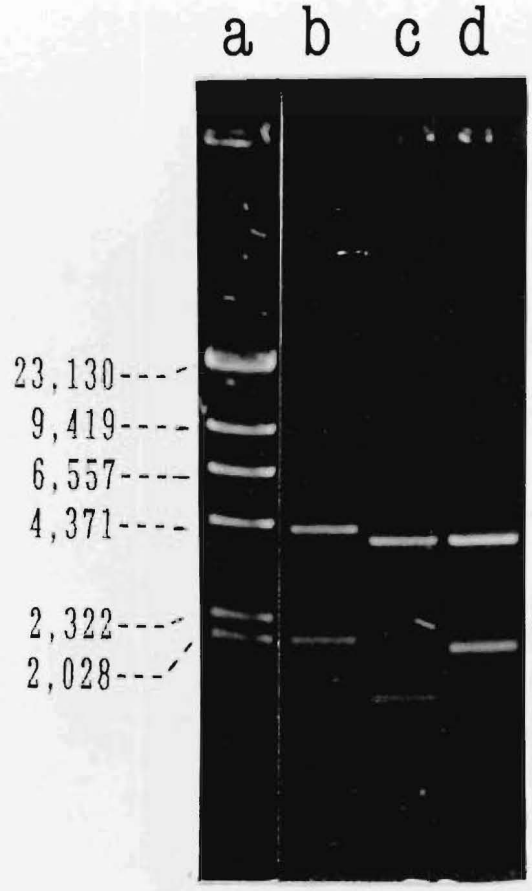
anfA
pANF135
E/H
H/B
pANF13

B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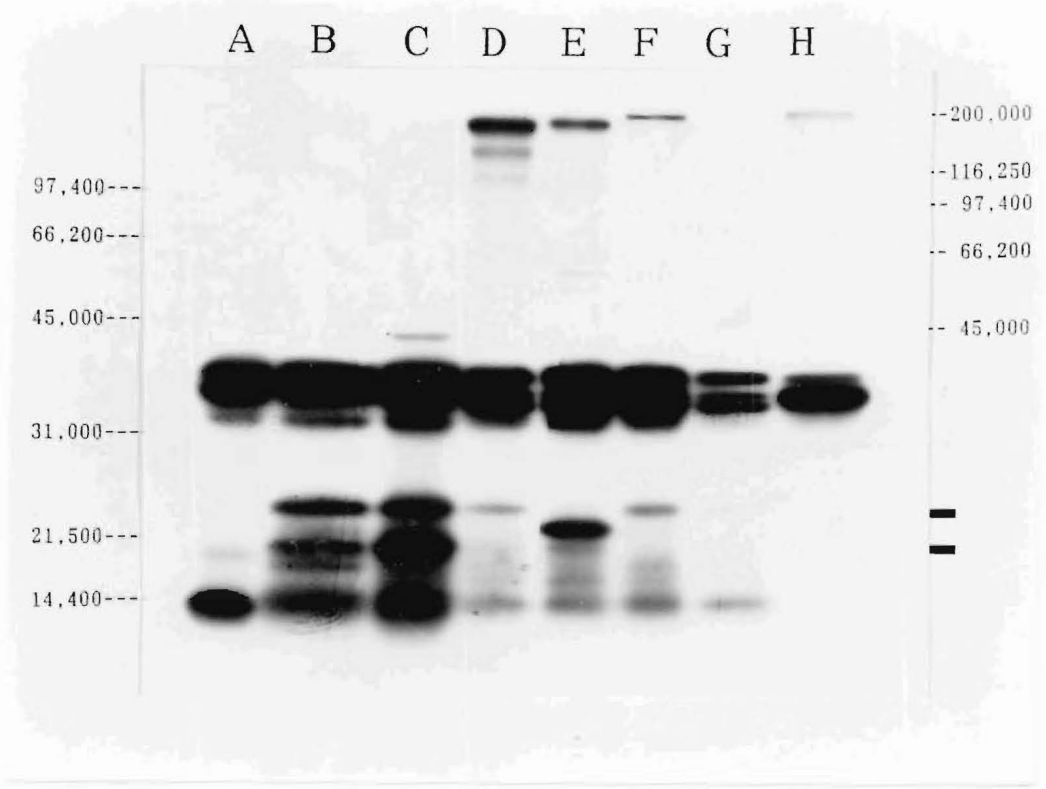

Fig. 5.4. Gene products of *amb2* locus and ß-galactosidase fusion proteins. SDS-PAGE of plasmid-mediated polypeptides expressed in minicells labelled with [^{35}S]methionine. Lanes: A) pBR322; B) pANF4 OriA; C) pANF4 Ori B; D) pMMU5; E) pMMU24; F) pMMU36; G) pMMU37; H) pMMU48. The location of mini-Mu insertions in the *amb2* locus is in Fig. 5.6. The pBR322 products are shown as a control; molecular sizes of standard proteins are marked in Daltons (Da). Electrophoretic conditions: 8 to 16% gradient polyacrylamide gel; the run was at 45 Volts for 16 hrs at room temperature.

transfer of these plasmids to *E. coli* P678-54T, the plasmid gene products were analyzed as previously described. Plasmid pANF22 expressed only the 25 kDa protein, AnfB (Fig. 5.2, lane C) while pANF135 exclusively synthesized the protein of 21 kDa, AnfA (Fig. 5.2, lane D). The deletion mutant pANF13 failed to produce the 21 kDa protein (Fig. 5.2, lane E). This plasmid is similar to pANF135, yet pANF13 lacks the 535 bp where the Tn*phoA* insertion point of the nonpathogenic UC24 mutant maps (Fig. 5.3). These results indicate that the 535 bp DNA fragment is essential for the synthesis of the 21 kDa protein (AnfA) and suggest that the loss of pathogenicity of the mutant UC24 is caused by the failure to synthesize this protein. The AnfA polypeptide was not observed in the protein profile of pANF13, but a 23 kDa peptide was produced by this plasmid (Fig. 5.2, faint band in lane E).

3.3) Mini-mu insertion mutagenesis and *lac* gene fusions

The genes encoding the proteins AnfA and AnfB have been partially mapped in the *amb2* locus. The *anfA* gene is located at right side of the 5.3 Kb DNA fragment, approximately 1.8 kb to the left of the *Bam*HI site. The *anfB* gene is at left side of the locus between the terminal *Bam*HI and *Hind*III restriction sites (Fig. 5.3). To map the promoters in the *amb2* locus and to create *anfA* and *anfB* mutants, Mini-Mu insertion mutagenesis was carried out.

The Mu phage is a transposable element that has been genetically manipulated by Castilho *et al.*, (1984) to be used as a mutagenesis system[1]. The mini-Mu element utilized in this work (MudII 1681) contains the structural genes of the *lac* operon and a selectable marker (Km^r), and can be used to form translational *lac* gene fusions (see Fig. 5.6). When the mini-Mu element inserts downstream a promoter and a translation signals, an enzymatically active hybrid ß-galactosidase will be expressed. This mutagenesis system (mini-Mu) permits the selection of Mu insertions in a high copy-number plasmid such as pBR322.

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[1] The bacteriophage Mu replicates by a process that involve transposition. While the transposition is repressed in the lysogenic state, it is induced by lytic growth. Foreign DNA sequences can be incorporated between the ends of the phage DNA, packaged into a viral particle and carried to a new cell and genomic locations. Segments of the Mu genome have been deleted and new genes (as the *lacZYA* operon) have been incorporated. The defective phage can be complemented with a helper Mu prophage. Transcription from a promoter outside the Mu insertion can proceed into the 116 bp of the Mu right end to express the *lac* genes (Castilho, *et al.*, 1984).

By means of this system, five mini-Mu insertions mutants in the *amb2* locus were isolated; two of them resulted in the expression of LacZ hybrid proteins with AnfA and AnfB proteins. Also, four tentative promoters signals were mapped and the direction of the transcription was determined. These results are described in the following sections.

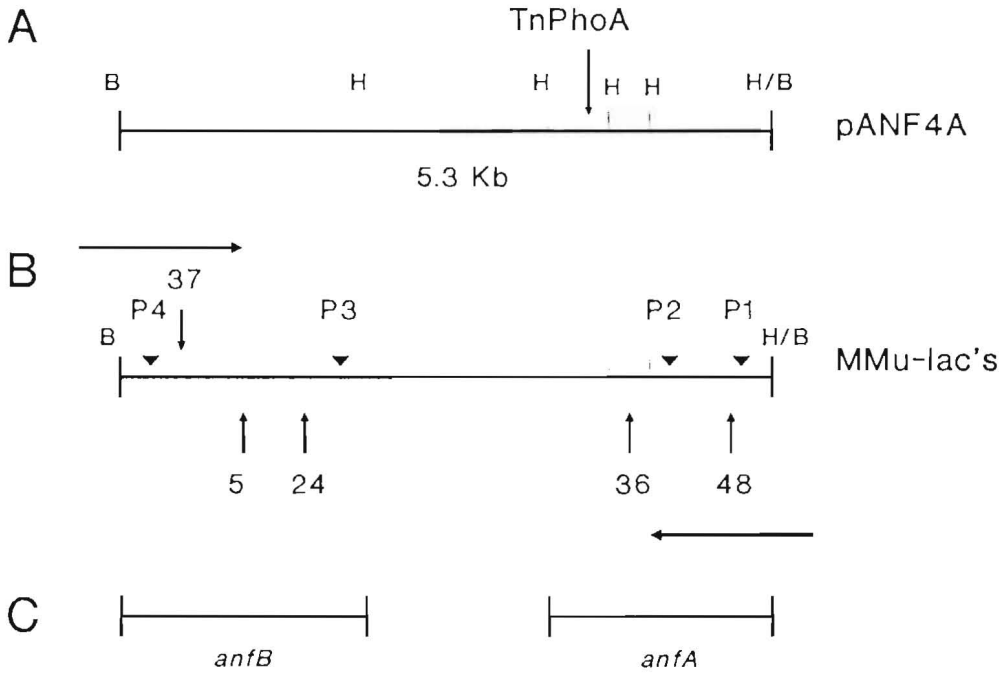
3.3.1) Construction of the mini-Mu mutants in the *amb2* locus and determination of transcription direction

Competent cells, *E. coli* strain POII 1681 (Castilho, *et al.*, 1984) which has inserted in the chromosome the mini-MudII-*lacZ* prophage and the complementing Mu *cts* prophage (Mu temperature-sensitive repressor), was transformed with pANF4 for the isolation of mini-Mu insertions into *amb2* locus. A single transformant selected by resistance to Ap and Km was obtained. Analysis by *Hind*III digestion of the plasmid and agarose gel electrophoresis confirmed the presence of the plasmid pANF4. After propagation and heat-induction for Mu lytic growth of the transformant, the lysate, carrying packaged duplicated plasmid mini-Mu insertions, was used for infection of the *E. coli* strain MH3497. This strain is Mu immune, so the phage replication is suppressed. Because it is $recA^+$ as well, the duplicated mini-Mu sequences undergoes homologous recombination and a plasmid with a mini-Mu insertion is generated.

Colonies grown on agar plates containing Ap, Km and X-gal were selected. Fifty colonies showing different levels of ß-galactosidase activity were screened by isolation of plasmid DNA, digestion with several restriction enzymes and electrophoresis. Five different *amb2*-mini-Mu insertion mutants were isolated and mapped (Fig. 5.5, part B and D). Insertions were distributed randomly along the 5.3 Kb DNA fragment, although certain clustering was observed. The other forty five isolates had the mini-Mu insertion in the vector DNA and were not further analyzed.

The insertion mutants namely pMMU37, pMMU5 and pMMU24 mapped into the *anfB* gene DNA region at approximately 0.6, 1.3 and 1.9 Kb from the left *Bam*HI site of the *amb2* locus (Fig. 5.5, part B). The orientation of the *lac* operon genes have been determined by restriction mapping (see Fig. 5.6) and hence the direction of transcription. Expression of the *lacZ* gene in a fusion requires that the orientation of this gene be the same as the orientation of the target gene. When the *lac* genes in this system are fused to another gene, as *anfB* and they are under the control of the

Fig. 5.5. Physical map of *amb2* locus and mini-Mu insertion mutants. Part A: restriction map of *amb2* locus in pANF4 OriA; the Tn*phoA* insertion site in mutant UC24 is indicated. Restriction enzymes: B, *Bam*HI; H, *Hind*III. Part B: Mini-Mu-*lac* insertion mutants; numbers and arrows indicate the insertion sites and transcription direction in pMMU5, pMMU24, pMMU36, pMMU48 and pMMU37; tentative regions of promoter signals are also indicated (P1 to P4). Part C: the regions corresponding to *anfA* and *anfB* genes are shown. Part D: 0.7% agarose gel electrophoresis of mini-Mu insertion mutants digested with *Hind*III. Lane: A) Lambda/*Hind*III; B) pMMU5; C) pMMU24; D) pMMU36; E) pMMU37; F) pMMU24; G) pMMU48; H) 1 Kb DNA Ladder (molecular size markers).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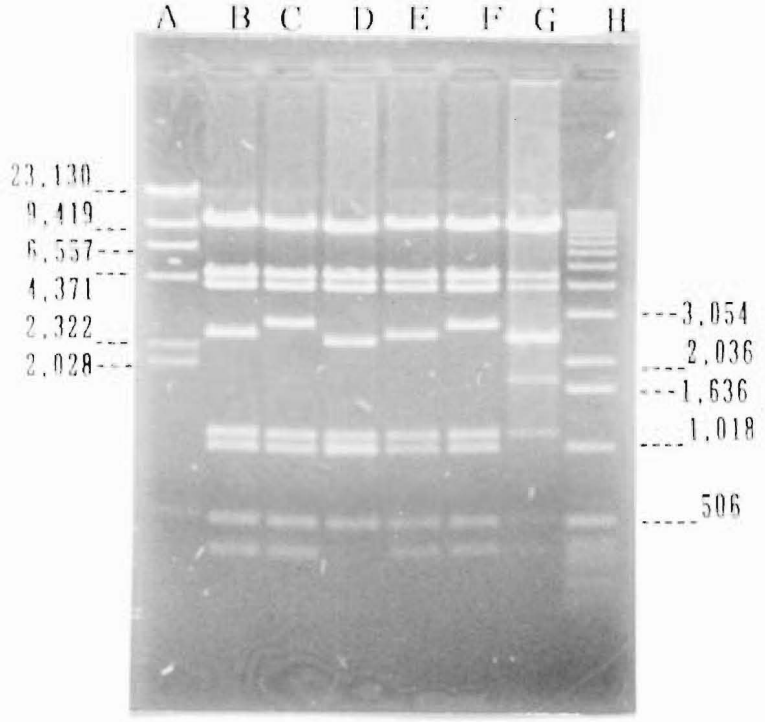
B
H
H
H
H
H/B
pANF4A
5.3 Kb
B
37
P4
P3
P2
P1
B
H/B
MMu-lac's
5
24
36
48
C
anfB
anfA

D


A
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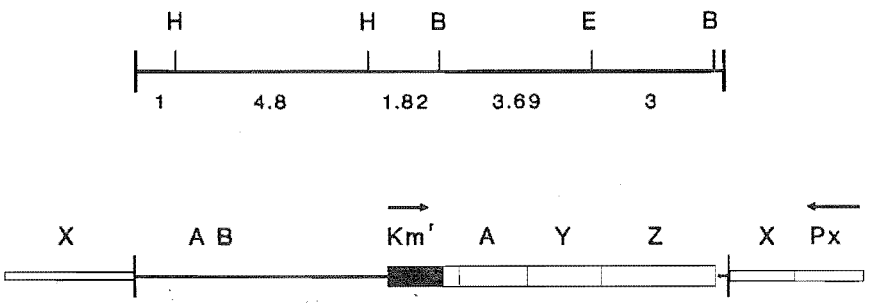


Fig. 5.6. Restriction map of Mini-MudII-*lacZ* prophage (Mu dII1681). The size of the prophage is 14.2 Kb and it confers Km^r. This mini-Mu element has a 116 bp segment from the Mu right end joined to *lacZ*. The *lacZ* gene has missed the translation initiation region and the first eight non essential *lacZ* codons. When this element integrates into structural genes in the appropriate direction and reading frame, transcription and translation from outside gene controlling regions can proceed across the 116 nucleotides from the right end of Mu into *lacZ* codons to form hybrid proteins that are enzymatically active for ß-galactosidase (Casadaban and Chou, 1984). The transposition-replication genes A and B are indicated on the left side of Mu. X represent an hypothetical gene X with promoter Px. Arrows show transcription direction. Numbers represent Kb. Restriction enzymes: E, *Eco*RI; B, *Bam*HI; H, *Hind*III (modified from Castilho, *et al.*, 1984).

promoter and controlling elements of such gene, the *lac* operon must have the same orientation as the *anfB* gene. Therefore, the direction of transcription is from right to left for the pMMU5 and pMMU24 mutants and the opposite direction for pMMU37 (Fig. 5.5, part B). On the other hand, the insertion point for the mutants pMMU36 and pMMU48 was located in the *anfA* gene region and they seem to be transcribed from right to left as well as pMMU5 and pMMU24 according with the *lac* gene orientation. The mutant pMMU36 was mapped approx 1.4 Kb from the right *Bam*HI site of the *amb2* locus and pMMU48 at 0.3 Kb from the same site (Fig. 5.5 part B).

3.3.2) Identification of promoters in the *amb2* locus

From these results, there are at least four possible DNA promoter sequences along the *amb2* locus. One seems to be controlling the *anfB* gene and therefore the expression of the ß-galactosidase of the mutants pMMU5 and pMMU24. This promoter named P3 might be located somewhere upstream of the first *Hind*III site about 2.1 kb from the *Bam*HI site (Fig. 5.5, part B). Other promoter signal (P2), seems to be in the *anfA* gene. The P2 sequences must be anywhere at right of the pMMU36 mutant insertion point relative with the map in Fig. 5.5, (part B). There is probably a different promoter signal (P1) regulating expression of *lacZ* in the mutant pMMU48 and it could collaborate in the transcription of both *anfA* and *anfB* genes. The last identified potential promoter (P4) lies close to the left terminal of the *amb2* fragment and controls the *lacZ* production in an opposite P1, P2 and P3 promoters direction. Due to its position and orientation, it is not associated with the AnfA and AnfB protein synthesis.

3.3.3) Determination of the *amb2-lac* gene fusions

To confirm the location of the genes encoding the AnfA and AnfB proteins and to identify the nature of *lac* fusions in the *amb2*-mini-Mu mutants, analysis of the gene products was carried out by minicells and SDS-PAGE.

The mini-Mu insertion mutants plasmids were introduced into *E. coli* strain P678-54T by electroporation since attempts by conventional transformation protocols were not successful for relatively big plasmids, in this case around 24 Kb. The presence of the expected plasmids in the Ap^r, Km^r transformants was confirmed by restriction analysis of the isolated plasmids. Gel electrophoresis of the minicells ^{35}S-

methionine labelled proteins is shown in the Fig. 5.4. A band about 28 kDa MW, close to the ß-lactamase proteins encoded by the vector pBR322 (see section 3.1), appears in the mini-Mu mutants (lanes D, E, F and G). This protein might be associated with the Km^r marker or with the Mu A and B genes necessary for transposition and replication of the mini-Mu element (Fig. 5.6). Lane F in the autoradiogram indicates that the pMMU36 mutant encodes the 25 kDa protein (AnfB) but fails to synthesize the 21 kDa protein (AnfA). In addition, it encodes a new protein band with size apparently larger than the reported 135 kDa MW for the wild type ß-galactosidase (Casadaban, *et al.*, 1980). This result confirms the position of the *anfA* gene at the right side of the *amb2* locus, in the 1.9 kb DNA insert in pANF1.3.5 (Fig. 5.3). In a similar way, the 25 kDa product is absent in the pMMU24 mutant and a smaller protein, about 23 kDa, is detected (Fig. 5.4, lane E). The origin of this protein is unknown, but probably it is a truncated protein. An AnfB-ß-galactosidase hybrid protein seems to be synthesized as well. This finding correlates with the mapping of *anfB* gene in the 2.1 DNA fragment cloned in pANF22 (Fig. 5.3). Lane D in the Fig. 5.4, shows the protein profile of the pMMU5 mutant. No loss of proteins was detected in this plasmid; both AnfA and AnfB proteins appear to be synthesized, though to a lesser degree than the hybrid ß-galactosidase protein appearing at top of the lane.

The mutant pMMU48 expressed only an apparent hybrid ß-galactosidase protein and the two polypeptides associated with the ß-lactamase of pBR322 (Fig. 5.4, lane H). One of these proteins seems to be overexpressed. The 28 kDa protein, AnfA and AnfB are not synthesized by the mutant.

Lane G in the Fig. 5.4 shows the protein profile of the mutant pMMU37. Both proteins AnfA and AnfB (very faint bands), and also a hybrid ß-galactosidase protein seem to be synthesized in this mutant. These proteins were evident at a longer exposure of the gel on the X-ray film (data not shown). Therefore, the insertion in the mutant pMMU37 had no apparent effect on the synthesis of AnfA and AnfB.

3.3.4) Qualitative scoring of ß-galactosidase activity of *amb2* gene fusions mutants

Plasmid mini-Mu insertion mutants were grown in LB-agar containing Ap and Km plus the histochemical stain X-Gal to qualitatively monitor the ß-galactosidase activity expressed by the mutants. The hydrolysis of X-Gal in the

presence of oxygen yields an insoluble blue dye. The indicator is very sensitive and even very low ß-galactosidase activity is detected. All five strains were phenotypically Lac^+ after 48 hours culture, although different levels of ß-galactosidase activity was observed (Fig. 5.7). The mutants pMMU24, pMMU36 and pMMU37 were low-level fusions showing low levels of ß-galactosidase activity (light blue color) in comparison with the pMMU5 and pMMU48 (strong blue color).

3.4) Bioassays with the *amb2* deletions mutants

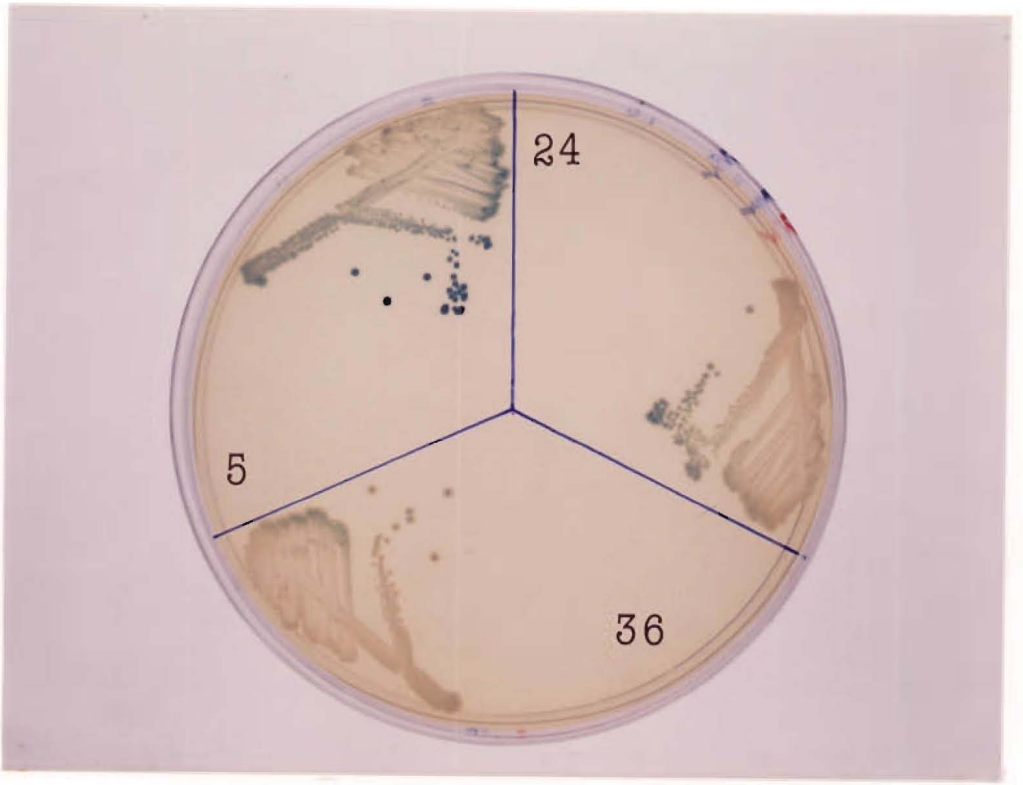
The molecular analysis of the *amb2* locus causing anti-feeding effect has revealed that the 5.3 Kb DNA is responsible for the synthesis of two polypeptides, AnfA and AnfB. The precise role of both proteins in the anti-feeding effect (AFE) is unknown. In order to determine the function of AnfA and AnfB, bioassays with larvae fed by nonpathogenic *S. entomophila* mutants carrying *anfA* and *anfB* genes were performed.

3.4.1) Cloning of the *amb2* deletion mutants into pLAFR3

The *anfA* and *anfB* genes were cloned in pBR322. Since *S. entomophila* nonpathogenic mutants UC24 and UC7 are Ap^r and Km^r, selection for pBR322 is difficult. Therefore, the *amb2* deletion mutants were cloned in pLAFR3. The Tc^r marker and the RP4 replicon (Pouwels, *et al.*, 1985) of this plasmid are suitable for the *S. entomophila* mutants.

The plasmids pANF135 bearing the *anfA* gene and pANF13 carrying a deletion mutant of the same gene (Fig. 5.3), were first doubled digested with *Bam*HI and *Eco*RI. The vector DNA was then cut with *Sal1* to avoid religation of insert DNA and pBR322. Ligation was carried out with *Bam*HI and *Eco*RI restricted pLAFR3. In addition, pANF22 digested also by *Bam*HI and *Sal*1 was ligated with *Bam*HI cut pLAFR3. *E. coli* DH5α was transformed with the ligation mixtures and Tc^r colonies were selected. Isolated plasmids from white colonies growing in the presence of X-gal were analyzed by restriction analysis. Three constructs were obtained with inserts about 1.9 kb corresponding to *anfA* gene, 1.4 kb related with an *anfA* deletion and 2.2 kb containing the *anfB* gene. The recombinant plasmids were named pENV135, pENV13 and pENV22. The plasmids were transferred to *S. entomophila* nonpathogenic mutants in order to test AFE by bioassays with insect larvae.

Fig. 5.7. ß-galactosidase activity of *amb2* gene fusion mutants. Five *amb2* mutants were obtained after Mini-MudII-*lacZ* insertion mutagenesis. *E. coli* strain MH3497 containing mutant plasmids were grown on LB-agar containing Ap, Km and X-Gal. After 48 hrs culture all mutants showed ß-galactosidase activity. Symbols: 5, pMMU5; 24, pMMU24; 36, pMMU36; 48, pMMU48; 37, pMMU37; C, control.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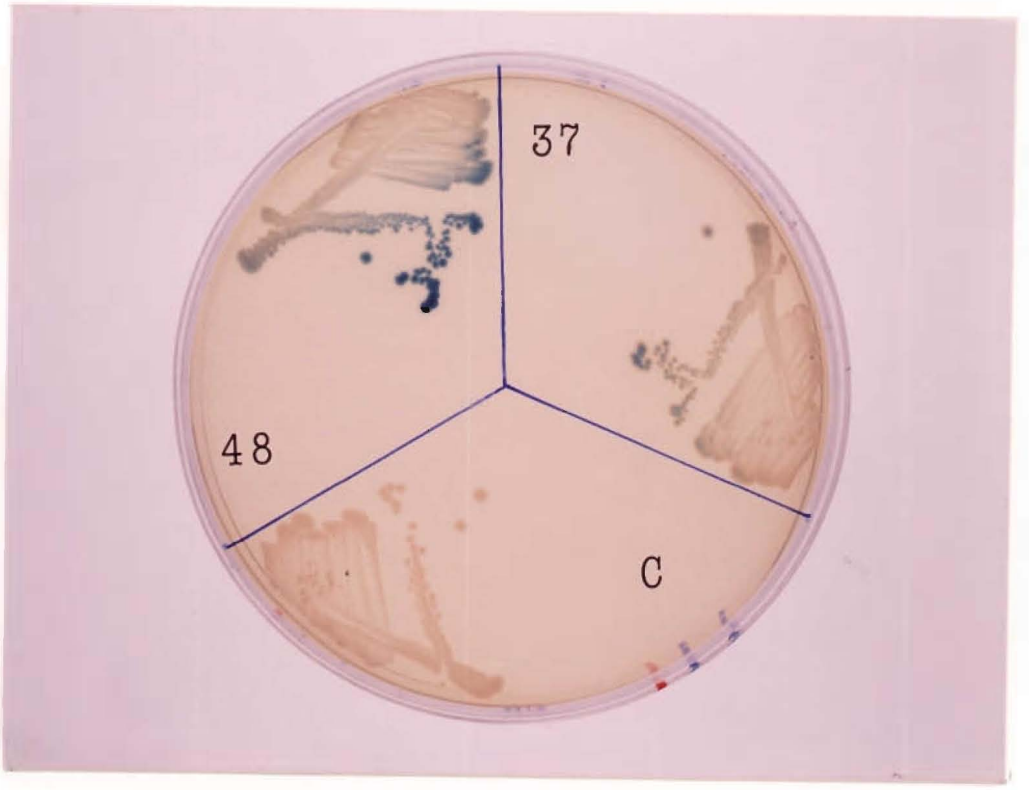
5
36


37
48
C

3.4.2) Bioassays with insect larvae

Plasmids pENV135, pENV13 and pENV22 were introduced to the nonpathogenic mutants UC7 and UC24 by electroporation. Colonies showing Ap^r, Km^r and Tc^r were selected to feed insect larvae. Bioassays were carried out as previously described and results are shown in Fig. 5.8 and 5.9. AFE was clearly observed in control groups, larvae fed with the pathogenic strain UC9 and the nonpathogenic strains UC7 and UC24 carrying *amb2* locus in pENV1 ($P< 0.001$). No AFE was observed with these strains bearing pLAFR3 as a negative control and the three *amb2* deletion mutants (pENV135, pENV13 and pENV22; Fig. 5.2; $P< 0.05$). This bioassay results confirmed the hypothesis that the presence of both the proteins AnfA and AnfB are essential for the expression of the anti-feeding effect.

3.5) DNA sequencing of the *anfA* gene

3.5.1) Sequencing strategy

DNA sequencing was performed by the dideoxy chain termination method of Sanger *et al.*, (1977) using the ^{T7}Sequencing^{TM} kit from Pharmacia LKB. Some sequencing data were obtained from Automated DNA sequencing service, Auckland.

Double stranded plasmid templates were used for sequencing, taking advantage of the pBluescript phagemid system. These plasmids have a polylinker region with multiple restriction sites which facilitates cloning of DNA fragments to be sequenced. Flanking the polylinker region, there are two polymerase promoters (T7 and T3) plus some DNA sequences from M13. These sites may be used for annealing primers to templates to start DNA polymerase reactions. Also, the plasmids have a *lacZ* gene located upstream of the promoters and linkers sites, hence, recombinant plasmids are easily selected on plates containing X-gal[2].

Therefore, DNA fragments to be sequenced were cloned into the pBluescript (KS M13-) phagemid. The strategy for cloning and sequencing is summarized in Fig. 5.10A. Briefly, pANF135 and pANF13 containing sequences of the *anfA* gene, were double digested with *Eco*RI and *Kpn*I. DNA fragments of 1.6 and 1.1 Kb

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[2] DNA inserts disrupt the coding region of the LacZ gene and bacteria containing recombinant plasmids grow as white colonies in the presence of X-Gal. Colonies with non-recombinant plasmids develop blue colour.

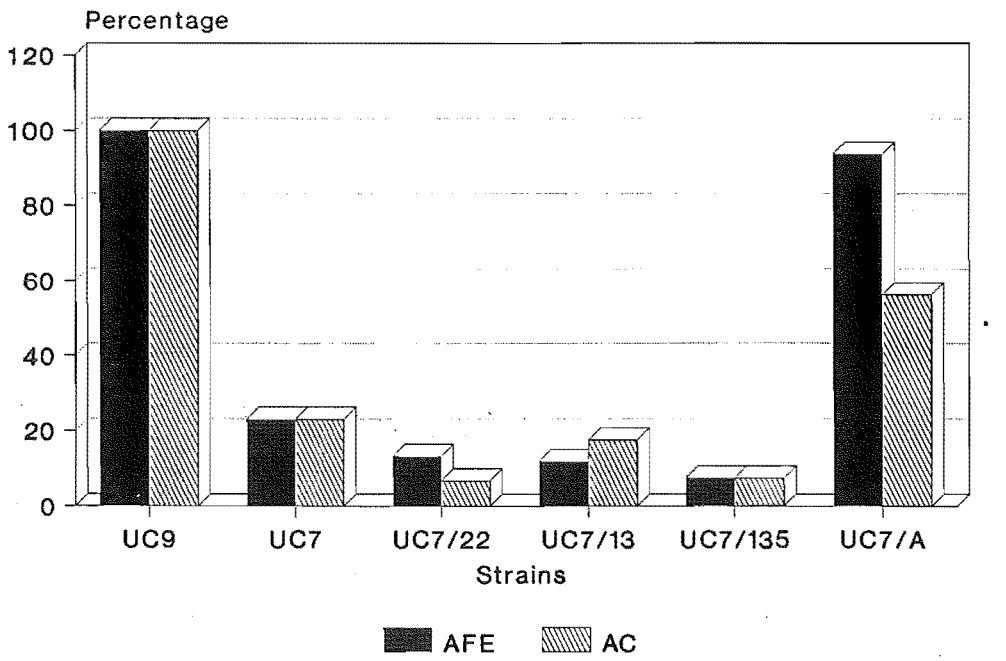

Fig. 5.8. Insect larvae bioassay of *S. entomophila* UC7 carrying *amb2* locus and deletion mutants. Plasmids pENV1 OriA (A), pENV22 (23), pENV13 (13) and pENV135 (135) were transferred to UC7 to test for anti-feeding effect (AFE) and amber coloration (AC). The positive control was the Path$^+$ strain UC9. The negative control was UC7 carrying the vector pLAFR3. AFE and AC are in percentage of larvae showing these amber disease symptoms after 7 days of inoculation.

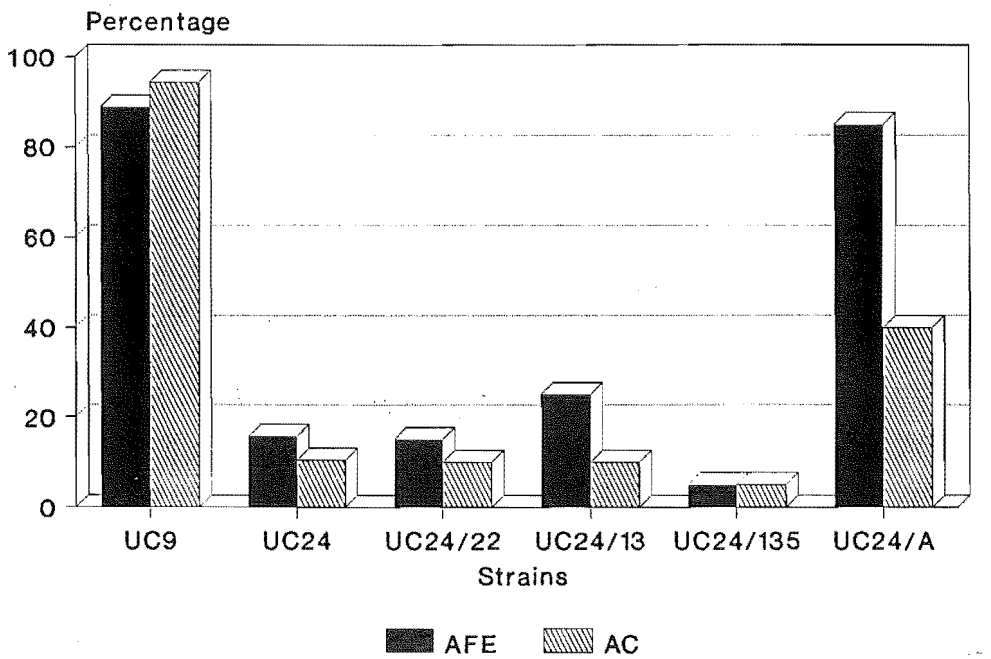

Fig. 5.9. Insect larvae bioassay of *S. entomophila* UC24 carrying *amb2* locus and deletion mutants. Plasmids pENV1 OriA (A), pENV22 (22), pENV13 (13) and pENV135 (135) were transferred to UC24 to test for anti-feeding effect (AFE) and amcer coloration (AC). The positivecontrol was the Path$^+$ strain UC9. The negative control was UC24 carrying the vector pLAFR3. AFE and AC are in percentage of larvae showing these amber disease symptoms after 7 days of inoculation.

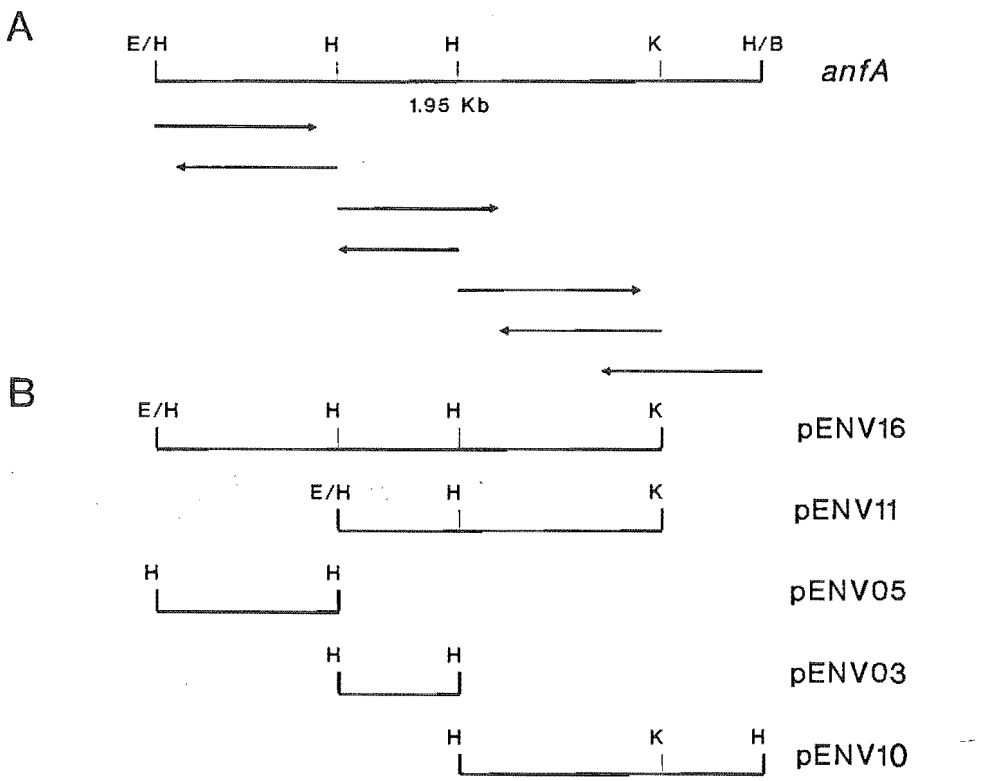

Fig. 5.10. Restriction map and sequencing strategy of *anfA* gene.

A) The map shows relevant restriction enzymes sites of *anfA*. The arrows indicate the direction and extent of sequencing from the different restriction sites of DNA inserts from the recombinant pBluescript plasmids figured in panel B.

B) Diagram showing the inserts in the plasmid constructs used for DNA sequence determination.

respectively, were isolated from a low melting point agarose gel. These fragments were ligated with pBluescript (KS M13-) also digested with *Eco*RI and *Kpn*I to create the plasmids pENV16 and pENV11 (Fig. 5.10B). The ligation mixtures were used to transform *E. coli* DH5α and white colonies were selected on agar plates containing Ap and X-Gal. The presence of recombinant plasmids were confirmed by DNA extraction and restriction analysis.

Nucleotide sequence was first determined in both direction of the cloned fragments, starting from the *Eco*RI and *Kpn*I sites in both plasmids. To extend the sequence, other plasmids were similarly constructed. Plasmid pANF135 was *Hind*III digested to isolate the DNA fragments of 0.5, 0.3 and 1 Kb. Each one of these fragments, was ligated separately with the vector pBluescript (KS M13-) previously digested with *Hind*III and CIP treated, to avoid religation of the vector plasmid. After ligation and transformation of *E. coli* DH5α, plasmids were purified from white colonies and confirmed by restriction analysis. Recombinant plasmids were named pENV05, pENV03 and pENV10 (Fig. 5.10B). Orientation of the inserts was determined by restriction analysis and by the previous sequencing data from pENV11 and pENV16. DNA inserts from the three plasmids were sequenced in both directions as delineated in Fig. 5.10A.

3.5.2) Nucleotide sequence of the *anfA* gene region and deduced amino acid sequence of the protein

The nucleotide sequence of the *anfA* gene region is shown in Fig. 5.11. The deduced amino acid sequence is also indicated. Sequencing data were analyzed by DNASIS™ (HIBIO, Hitachi America, LTD) and Genesys (Bottonley, W., 1987, Australia) software.

Two open reading frames (ORF) of 575 and 323 bp were found along the *anfA* gene(s) region. These ORFs should generate two translation products with calculated molecular weight of 22,267 and 11,967 kDa, named $AnfA_1$ and $AnfA_2$ respectively. No putative Shine-Dalgarno[3] sequences were found close to the ATG initiation codons at positions 873 and 1597. However, the purine rich segments -

[3] Shine-Dalgarno sequence is part or all of the polypurine sequence AGGAGG located on bacterial mRNA just prior to an AUG initiation codon; is complementary to the sequence at the 3' end of 16S rRNA; involved in binding of ribosome to mRNA (Lewin, 1987).

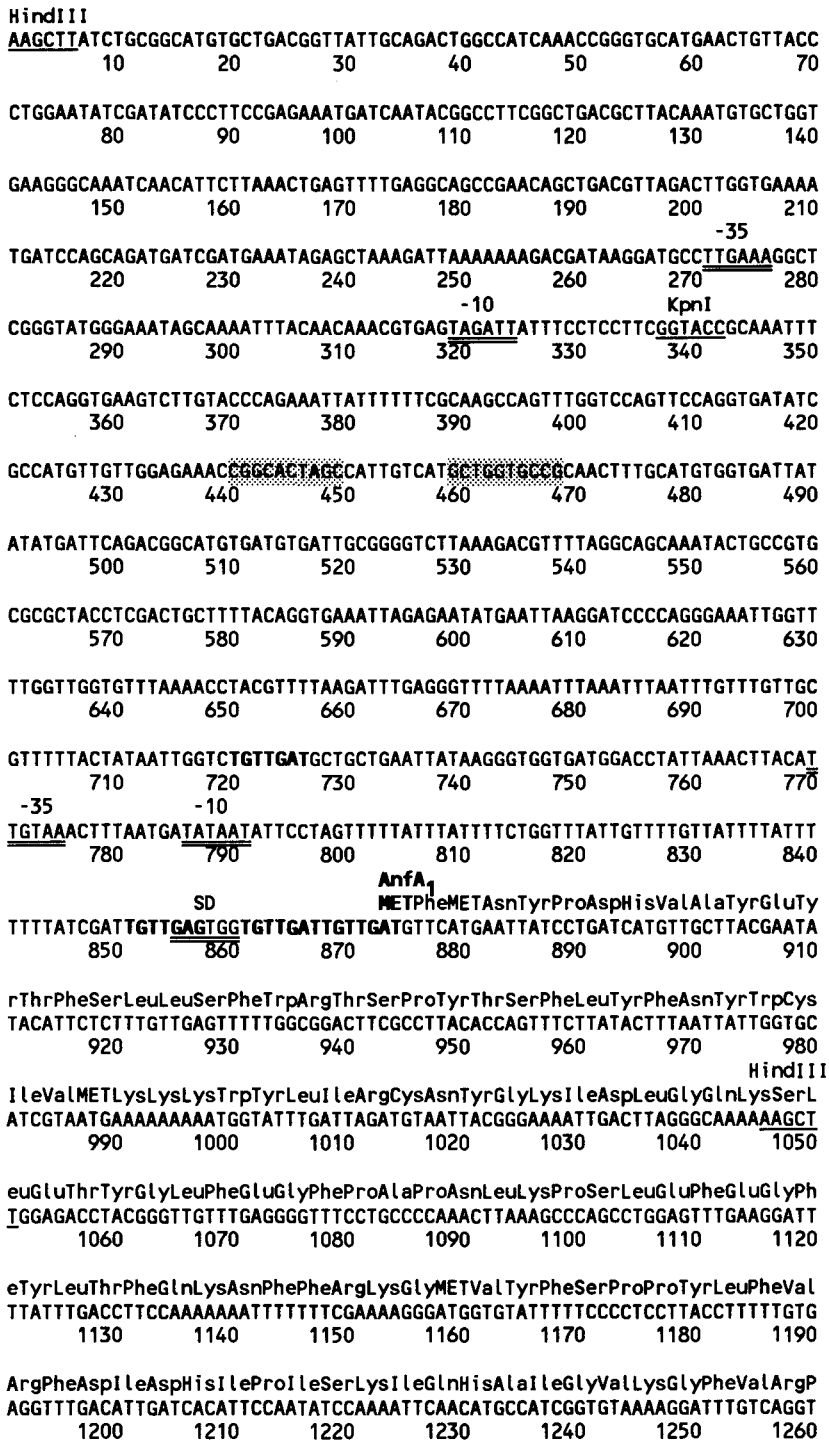
HindIII
AAGCTTATCTGCGGCATGTGCTGACGGTTATTGCAGACTGGCCATCAAACCGGGTGCATGAACTGTTACC
10 20 30 40 50 60 70
CTGGAATATCGATATCCCTTCCGAGAAATGATCAATACGGCCTTCGGCTGACGCTTACAAATGTGCTGGT
80 90 100 110 120 130 140
GAAGGGCAAATCAACATTCTTAAACTGAGTTTTGAGGCAGCCGAACAGCTGACGTTAGACTTGGTGAAAA
150 160 170 180 190 200 210
-35
TGATCCAGCAGATGATCGATGAAATAGAGCTAAAGATTAAAAAAAGACGATAAGGATGCCTTGAAAGGCT
220 230 240 250 260 270 280
-10 KpnI
CGGGTATGGGAAATAGCAAAATTTACAACAAACGTGAGTAGATTATTTCCTCCTTCGGTACCGCAAATTT
290 300 310 320 330 340 350
CTCCAGGTGAAGTCTTGTACCCAGAAATTATTTTTTCGCAAGCCAGTTTGGTCCAGTTCCAGGTGATATC
360 370 380 390 400 410 420
GCCATGTTGTTGGAGAAACCGGCACTAGCCATTGTCATGCTGGTGCCGCAACTTTGCATGTGGTGATTAT
430 440 450 460 470 480 490
ATATGATTCAGACGGCATGTGATGTGATTGCGGGGTCTTAAAGACGTTTTAGGCAGCAAATACTGCCGTG
500 510 520 530 540 550 560
CGCGCTACCTCGACTGCTTTTACAGGTGAAATTAGAGAATATGAATTAAGGATCCCCAGGGAAATTGGTT
570 580 590 600 610 620 630
TTGGTTGGTGTTTAAAACCTACGTTTTAAGATTTGAGGGTTTTAAAATTTAAATTTAATTTGTTTGTTGC
640 650 660 670 680 690 700
GTTTTTACTATAATTGGTCTGTTGATGCTGCTGAATTATAAGGGTGGTGATGGACCTATTAAACTTACAT
710 720 730 740 750 760 770
-35 -10
TGTAAACTTTAATGATATAATATTCCTAGTTTTTATTTATTTTCTGGTTTATTGTTTTGTTATTTTATTT
780 790 800 810 820 830 840
AnfA1
SD METPheMETAsnTyrProAspHisValAlaTyrGluTy
TTTTATCGATTGTTGAGTGGTGTTGATTGTTGATGTTCATGAATTATCCTGATCATGTTGCTTACGAATA
850 860 870 880 890 900 910
rThrPheSerLeuLeuSerPheTrpArgThrSerProTyrThrSerPheLeuTyrPheAsnTyrTrpCys
TACATTCTCTTTGTTGAGTTTTTGGCGGACTTCGCCTTACACCAGTTTCTTATACTTTAATTATTGGTGC
920 930 940 950 960 970 980
HindIII
IleValMETLysLysLysTrpTyrLeuIleArgCysAsnTyrGlyLysIleAspLeuGlyGlnLysSerL
ATCGTAATGAAAAAAAAATGGTATTTGATTAGATGTAATTACGGGAAAATTGACTTAGGGCAAAAAAGCT
990 1000 1010 1020 1030 1040 1050
euGluThrTyrGlyLeuPheGluGlyPheProAlaProAsnLeuLysProSerLeuGluPheGluGlyPh
TGGAGACCTACGGGTTGTTTGAGGGGTTTCCTGCCCCAAACTTAAAGCCCAGCCTGGAGTTTGAAGGATT
1060 1070 1080 1090 1100 1110 1120
eTyrLeuThrPheGlnLysAsnPhePheArgLysGlyMETValTyrPheSerProProTyrLeuPheVal
TTATTTGACCTTCCAAAAAAATTTTTTTCGAAAAGGGATGGTGTATTTTTCCCCTCCTTACCTTTTTGTG
1130 1140 1150 1160 1170 1180 1190
ArgPheAspIleAspHisIleProIleSerLysIleGlnHisAlaIleGlyValLysGlyPheValArgP
AGGTTTGACATTGATCACATTCCAATATCCAAAATTCAACATGCCATCGGTGTAAAAGGATTTGTCAGGT
1200 1210 1220 1230 1240 1250 1260

Fig. 5.11. Nucleotide sequence of the *S. entomophila anfA* genes and deduced amino acid sequence of the $AnfA_1$ and $AnfA_2$ proteins. The DNA sequence is represented from the 5' end of the sequenced fragment. Relevant restriction sites are underlined. The proposed -35 and -10 boxes, as well as the potential SD sequence are double underlined. The tandem repeat sequences of seven bp are in bold letters. The hydrophobic motif associated with the Domain 2 of δ-endotoxins of *B. thuringiensis* is in bold and overlined. Shadowed sequences represent a palindrome.

GAGTGG- located 12 bp upstream the ATG codon on the $anfA_1$ gene and the -GGGGACA- sequence, laying at 29 bp from the proposed initiation codon in $anfA_2$, might act as the ribosome binding site to initiate protein synthesis (Rosenberg and Court, 1979).

Upstream from the $anfA_1$ open reading frame, at position 786, there is a putative -10 (TATAAT) sequence[4]. There is also a likely -35 (TTGTAA)[5] region at position 770. Similar sequences were also found upstream from the $anfA_2$ open reading frame. The -10 (TTTAAT) sequence is located at base 1533 and the -35 (TTGCCA) at base 1503.

Possible Fur-binding sites were found in both promoter regions of $anfA_1$ and $anfA_2$ (Fig. 5.12). These regions presented homologies of 58% and 68% respectively, related with the putative Fur-binding consensus sequences reported for *E. coli* (Calderwood and Mekalanos, 1987). The proposed sequence in the promoter of $anfA_1$ overlaps with the possible -10 box, unlike the Fur-binding site for $anfA_2$, which was found downstream of the -10 region.

Three direct tandem repeats of seven bp sequence -TGTTGAT- were found at position 851, and once again at site 719, in the promoter region of the $anfA_1$ gene, very close of the ATG initiation codon of the corresponding protein. This region is 86% homologous to the DNA sequence -TTTTGAT-, which is recognized by the trans-acting transcription regulator ToxR (Miller, *et al.*, 1987). This protein is a master regulator required for the production of multiple virulence factors in *V. cholerae* (Betley, *et al.*, 1986; DiRita, 1992).

A sequence, 59% homologous to the consensus CAP-binding site -AAATGTGATCTAGATCACATTT- (Botsford and Harman, 1992), was also found 10 bp upstream of the proposed -35 box of the $anfA_2$ gene. A 22-bp palindromic sequence with 91% homology with the consensus may be formed with 15 bp downstream from the first dyad of the symmetry (Fig. 5.13).

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[4] The Pribnow box is the sequence centred about 10 bp before the startpoint of bacterial genes. It is a part of the promoter especially important in binding RNA polymerase (Lewin, 1987).

[5] -35 is a consensus sequences -TTGACA- situated ~35 bp upstream of the start point of bacterial genes (Lewin, 1987).

Fig. 5.12. Possible Fur-binding site in the proposed promoter regions of the *anfA* genes. Vertical lines indicate matches with the reported consensus sequence (Calderwood and Mekalanos, 1987). Numbers indicate co-ordinates from the nucleotide sequence displayed in the Fig. 5.11.

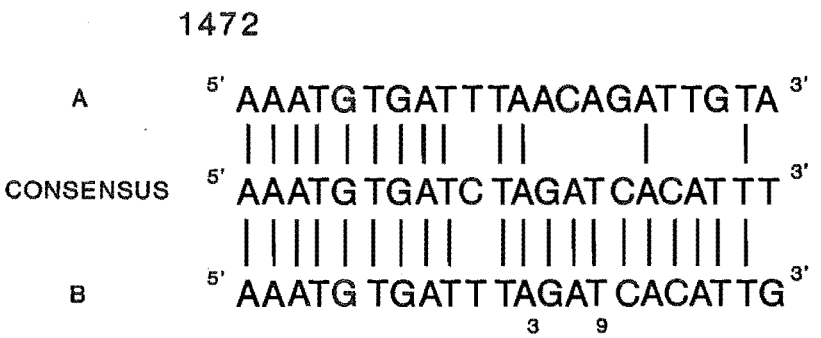

Fig. 5.13. CRP (CAP)-binding consensus sequence aligned with nucleotides from the proposed promoter region of *anfA*$_2$ gene. Vertical lines show matches with regions 59% (A) and 91% (B) homologous to the consensus sequence (Botsford and Harman, 1992). Numbers indicate co-ordinates from the nucleotide sequence displayed in the Fig. 5.11.

The National Centre for Biotechnology Information (NCBI) BLAST (Altschul, *et al.*, 1990) network service was used for DNA and protein homology searching with other published sequences. No significant homology was found with any other nucleotide or amino acid sequence. However, comparison of the deduced amino acid sequence of the $AnfA_1$ protein with sequences from a conserved domain (Block 2) of the δ-endotoxins of *Bacillus thuringiensis*, revealed 50% homology in a hydrophobic motif important for their toxic activity (Pereyra-Alférez, *et al.*, 1992). The percentage of homology was 78% when other hydrophobic amino acids present in the sequence were considered equivalent (Fig. 5.14).

Other tentative promoter signals were identified in the *anfA* gene region; the proposed -10 sequence (TAGATT) is at position 319 and the -35 region (TTGAAA) is located at base 271. Another possible Fur-binding sequence (AATAGAGCTAAAGATTA) was also identified upstream from the -35 sequence (at base 233; Fig. 5.11). The percentage of homology with the putative sequences reported for *E. coli* (Lewin, 1987; Calderwood and Mekalanos, 1987) were 66%, 83% and 58% respectively.

4) DISCUSSION

4.1) Gene products of *amb2* locus

The molecular analysis of the *amb2* locus causing anti-feeding effect has revealed that the 5.3 Kb DNA fragment is responsible for the synthesis of two polypeptides of 21 and 25 kDa. It was shown that these proteins are encoded by the *anfA* and *anfB* genes. The DNA region encoding both proteins has been mapped by minicells protein analysis of deletion mutants. The plasmid pANF135 exclusively synthesized the AnfA protein and pANF22 only expressed the AnfB peptide (Fig. 5.2). These results have been confirmed with similar analysis of the *amb2* mini-Mu insertion mutants. The insertion in pMMU36 eliminated the synthesis of AnfA protein whereas the insertion in pMMU24 suppressed the synthesis of the AnfB peptide. The transcription direction of both *anfA* and *anfB* genes proceed from right to left, relative to the map in Fig. 5.5 (part B).

Analysis in minicells of the gene products of *amb2* showed the production of two proteins of 17 and 42 kDa, in addition to AnfA and AnfB. The former was synthesized by pANF4 only in orientation A and the latter was produced only in

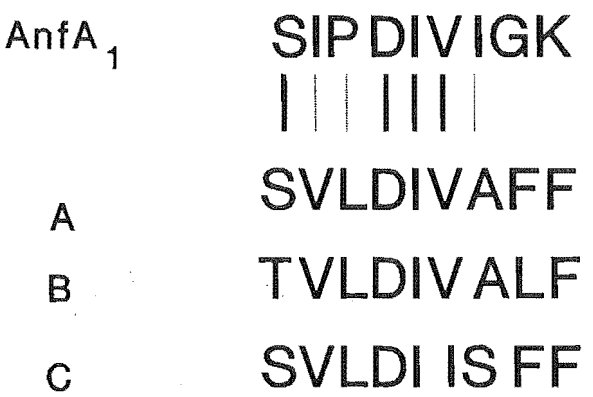

Fig. 5.14. Alignment of the amino acid sequences of a hydrophobic motif (Domain 2) present in the Cry protein family of δ-endotoxins of *B. thuringiensis* with sequences from AnfA$_1$ protein. Vertical lines show matches with identical amino acids. Dotted lines indicate matches with equivalent hydrophobic amino acids. A) *B. thuringiensis* HD-68 (Höfte, *et al.*, 1990); B) *B. thuringiensis aizawai* EG 6346 (Chambers, *et al.*, 1991); C) *B. thuringiensis kenyae* (Visser, *et al.*, 1990).

orientation B (Fig. 5.4, lanes B and C respectively). Therefore, a vector promoter appears to be involved in their synthesis. A promoter signal upstream of the region coding for Tc^r has been identified in pBR322 (Stüber and Bujard, 1981) and it is only inactivated for insertions into the *Hind*III site. The *amb2* locus has been cloned into the *Bam*HI restriction site, hence this vector promoter seems to be directing transcription for the 42 and/or 17 kDa proteins. On the other hand, several other promoter signals have been reported downstream from the Ap^r genes and the replication origin signal in pBR322 which could similarly collaborate for the expression of those proteins. The influence of *amb2* orientation on the synthesis of both proteins, could be related to the presence or absence of translation signals upstream from the protein coding sequence. Similar effect of vector sequences on minicells plasmid expressed proteins has been observed (Labigne-Roussel, *et al.*, 1985).

Analysis of *amb2* deletion derivatives showed that a 23 kDa peptide was originated from pANF13 instead of the *anfA* product (Fig. 5.2, lane E). A different reading frame might be present in this plasmid and probably it is used when the AnfA protein is not synthesized since the 23 kDA peptide is neither produced by pANF135 nor by the complete *amb2* sequence in pANF4 (Fig. 5.2, lane D and B, respectively). However, results from the DNA sequencing of the *anfA* gene (section 3.5) showed a termination codon in the 535 bp DNA fragment, which is not present in pANF13 (Fig. 5.3). Therefore, with no translation termination signal in the insert of pANF13, a larger protein than AnfA (21 kDa) was produced.

Five mini-Mu insertion mutants in the *amb2* locus were obtained and analyzed by expression in minicells and SDS-PAGE. The pMMU36 mutant failed to synthesize the AnfA protein, but produced a new polypeptide appearing in the migration region of the gel corresponding to ß-galactosidase (Fig. 5.4, lane F). This high molecular weight protein appears to be hybrid ß-galactosidase protein. This explains the disappearance of the 21 kDa protein (AnfA), which seems to be fused to the ß-galactosidase NH_2 terminal. Similarly, the insertion in pMMU24 repressed the production of AnfB and a hybrid ß-galactosidase protein was also synthesized. On the contrary, the insertion in the mutant pMMU5 did not abolish the expression of *anfA* and *anfB* suggesting that the first 1,000 bp to the left of the *amb2* DNA fragment are not related to them. An apparent ß-galactosidase hybrid protein is present, probably as a result of an open reading frame unrelated to the AnfB protein synthesis. The same hypothetical open reading frame could be responsible for the

presence of the 23 kDa peptide present in lane E, produced instead of the 25 kDa product. These insertions, pMMU5 and pMMU24 caused a reduction in the synthesis of the AnfA protein, whereas that of AnfB was unchanged and the 23 kDa protein appears to be overexpressed (lane D and E respectively). The reason for these observations is unknown at this stage.

The insertion in the mutant pMMU48 seems to have suppressed or diminished the synthesis of both AnfA and AnfB proteins (lane H). This polar effect on the level of synthesis of these proteins suggests the existence of a large transcriptional unit arising from the right to the left side of *amb2* locus according to the genetic map shown in Fig. 5.5 (part B). Important regulatory sequences could be present upstream of the insertion site of this mutant. Besides, a high level of expression of a ß-galactosidase hybrid protein is produced (section 3.3.4 and Fig. 5.7). However, the synthesis of the 28 kDa protein associated with the mini-Mu element (see Fig. 5.4), was also suppressed in this mutant. Therefore, an alternative explanation of the suppression of some proteins due to the insertion in pMMU48 is that the promoter P1 (Fig. 5.5, part B) might compete with the other promoters in the plasmid for initiation of transcription by RNA polymerase. Evidence of this hypothesis is that even the unprocessed form of ß-lactamase seems to be reduced, and a protein(s) of about 30 kDa is overexpressed. This protein band might contain the *lacY* permease and the *lacA* transacetylase, whose molecular weights are around 30 kDa (Lewin, 1987). Strong ß-galactosidase activity was observed in the mutant pMMU48 (Fig. 5.7) and this activity was stronger than the one observed in pMMU36 and pMMU24 (Fig. 5.7), which supports such hypothesis. Competition for RNA polymerase in minicells has been observed in the expression of the *Streptococcus mutants asd* (aspartate-semialdehyde dehydrogenase) gene in pBR322 (Jagusztyn-Krynicka, *et al.*, 1982). The possibility that the expression of ß-galactosidase in pMMU48 responds to vector promoter and translation signals is not excluded.

On the other hand, no loss of proteins was detected in the mutant pMMU37 (Fig. 5.4, lane G). In agreement with results derived from pMMU5 (lane D) this finding suggests no relationship of the first 600 bp at the left of *amb2* locus with the synthesis of AnfA and AnfB proteins. The expression of a probably hybrid protein was observed in this mutant (faint band in lane G), whose expression might be dependent on vector transcription and/or translation signals upstream the *Bam*HI site of pBR322.

The ß-galactosidase activity expressed by pMMU24, pMMU36 and pMMU37 was very low, contrary to the activity observed in pMMU5 and pMMU48. Apparently, low level expression of enzymatic activity might be due to wrong phase of the *lacZ* codons resulting in low level activity from translation frameshift or restarting in another open reading frame (Casadaban and Chou, 1984). An alternative explanation is that in the fusions of pMMU24 and pMMU36, the hybrid LacZ is likely directed to the cellular envelope. In this condition, the enzymatic activity is decreased dramatically (Slauch and Silhavy, 1991).

Bioassays with the *amb2* deletion derivatives allowed the individual evaluation of AnfA and AnfB on amber disease. No AFE was observed in larvae fed with the mutants UC24 and UC7 carrying pENV135 which expresses exclusively AnfA and pANF22 that only produce AnfB. Therefore, both AnfA and AnfB proteins and/or sequences contained in the full *amb2* locus seem to be essential for the AFE. On the other hand, it has been demonstrated that the Tn*phoA* insertion site in the mutant UC24 maps into the *anfA* gene(s) region (Chapter V). Therefore, if both proteins are essential and the *anfB* gene is intact in UC24, it was expected that such gene in the plasmid pENV135 would restore the AFE in that mutant. However, this did not happen in the actual experiment. This result confirms that both proteins are essential for the AFE and suggests that they can not be complemented in trans. The reason for this inability might be that the functional molecule is a multimeric form and this is unavailable in the mutational background. In addition, this result is in agreement with the hypothesis that the *amb2* locus seems to work in one transcriptional unit (see clause 3.3.3) with a strong promoter (P1) at the right of the locus (Fig. 5.5).

The *amb2-lacZ* fusion strains and the different levels of enzymatic activity produced might be used to score for environmental factors that stimulate *lacZ* expression in order to characterize the regulation of *amb2* genes.

4.2) DNA sequencing

The nucleotide sequence of a 1990 bp DNA fragment has been determined. The sequence confirmed previous results obtained by minicells analysis of the *amb2* locus. An ORF of 575 bp ($AnfA_1$) correlates with the AnfA protein of calculated MW of 21 kDa observed in the Fig. 5.2 and 5.4. The deduced MW obtained from the sequencing data of this protein was 22,267 kDa. The difference between the two sets of information suggests a possible processing step in this protein. Also, the proposed

P2 promoter region directing the synthesis of the hybrid ß-galactosidase protein in the insertion mutant pMMU36 (Fig. 5.4 and 5.5) is consistent with the -10 and -35 sequences found upstream from the start point of the $AnfA_1$ protein (Fig. 5.11).

A second ORF was identified beginning at position 1597, with a predicted MW of 11,967 kDa ($AnfA_2$). However, no protein of this size was observed in SDS-PAGE of minicells carrying either pANF4 or pANF135 (Fig. 5.4 and 5.2). This might be the result of two possibilities: i) that the 11,967 kDA protein is poorly synthesized by the bacteria in comparison with $AnfA_1$ and AnfB, and therefore it is not detected in the autoradiograph or, ii) a deficient resolution of small size proteins in the 8 to 16% gradient SDS-PAGE.

Three regulatory consensus sequences seem to be present along the promoter region of both proteins. The Fur-binding site of *E. coli* determines the union of the regulatory protein Fur, causing repression of gene transcription in the presence of enough iron in the medium (Schaffer, *et al.*, 1985; Calderwood and Mekalanos, 1987; 1988). Conversely, there is an increased gene expression under low iron availability. Virulence determinants in several pathogenic bacteria, seem to be regulated by iron and therefore they are dependant on Fur-like regulators. These include Shiga-like toxin I of *E. coli* (Calderwood and Mekalanos, 1987), diphtheria toxin of *Corynebacterium diphtheriae* (Boyd, *et al.*, 1990), the cytolysin of *Vibrio vulnificus* (Wright, *et al.*, 1985), the hemolysin of *S. marcescens* (Poole and Braun, 1988) and the outer membrane proteins of *Vibrio anguillarum* and *V. cholerae* (Actis, *et al.*, 1985; Goldberg, *et al.*, 1990). In addition, it has been demonstrated that the *S. marcescens* hemolysin determinant can be regulated by the product of the *fur* locus of *E. coli* (Poole and Braun, 1988). This fact implies that a similar Fur-like function operates in *Serratia* spp. Hence, it is not surprising that some virulence genes in *S. entomophila* may be regulated, at least in part, by iron and Fur-like regulators. The presence of Fur-binding sites in the promoter region of the AnfA proteins strongly suggest that these genes might be regulated by iron.

Another possible transcription regulatory sequence was identified in the promoter region of the $anfA_1$ gene. This is the ToxR-binding site found in promoters of well characterized virulence determinants of *V. cholerae*. The genes of the cholera toxin (Miller and Mekalanos, 1985), the toxin corregulated pilus Tcp (Taylor, *et al.*, 1987), the accessory colonization factor Acf and the 17 *tag* genes (Peterson and Mekalanos, 1988), are all part of a regulon that is coordinately

regulated by the transcriptional activator ToxR. This regulator is part of the two-component family of transcriptional activators in prokaryotes. This family includes the regulators OmpR, VirG, PhoP and PhoB (Albright, *et al.*, 1989; Miller, *et al.*, 1987; Ottemann, *et al.*, 1992). ToxR recognizes and binds to the sequence -TTTTGAT-, which is tandemly repeated 3-8 times upstream of the cholera toxin transcriptional start site (Miller, *et al.*, 1987). A homologous sequence (86%) was found tandemly repeated 3 times, very close to the proposed ATG initiation codon of the $anfA_1$ gene. This finding suggests that a similar regulatory machinery to that reported in *V. cholerae*, might control virulence genes in *S. entomophila*, including the *anfA* genes.

Analysis of the nucleotide sequence of the proposed promoter region of the $anfA_2$ gene, revealed certain homology with the 22 bp CRP(CAP)-binding site of *E. coli*. CRP is a cAMP receptor protein, which conforms a regulatory complex involved in multiple bacterial functions (Botsford and Harman, 1992). The bacteria respond to the presence of glucose by regulating the intracellular concentration of cAMP. The complex CRP-cAMP regulates the expression of many catabolic operons. In addition, it is also involved in other functions, not directly related to catabolism. These include the iron uptake regulon (De Lorenzo, *et al.*, 1988), flagellum synthesis (Kutsukake, *et al.*, 1990), enterotoxin production (Martinez-Cadena, *et al.*, 1981; Gilbert, *et al.*, 1989) and fimbriae expression (Eisenstein *et al.*, 1981; Eisenstein and Dodd, 1982; Schmoll, *et al.*, 1990; Göransson, *et al.*, 1989). Virulence determinants in *Salmonella typhimurium* seem to be dependent on the CRP-cAMP complex (Curtiss and Kelley, 1987). It is likely that virulence genes in *S. entomophila* may be controlled, at least in part, by this regulatory system. It has been suggested that expression of fimbriae (Chapter II) and proteases (Chapter III) in *S. entomophila* might be directly or indirectly regulated by the intracellular levels of cAMP. The presence of CRP-binding sites in the promoter region of the $anfA_2$ gene is an evidence of regulation of this virulence gene by the CRP-cAMP system.

Analysis of the sequencing data indicated that $AnfA_1$ and $AnfA_2$ are novel proteins, since no significant homology was found between any other reported DNA and amino acid sequences. However, some homology was observed between a hydrophobic region present on the Domain 2 of δ-endotoxins of *B. thuringiensis*, and the $AnfA_1$ protein. This is relevant, since the hydrophobic motifs in the δ-endotoxins seem to be very important for their entomocidal activity. These toxins are crystal proteins, toxic to larvae of diverse groups of insects, conforming the Cry

protein family. The toxicity of these proteins appear to be related with the production of pores on the cell membrane of the insect midgut, causing cell lysis. Five conserved domains have been identified among the toxins of the Cry family (Höfte and Whiteley, 1989; Lerecleus, *et al.*, 1989) and have been implicated in toxicity. Hydrophobic regions appear to be very important for the three dimensional structure of the CryIII A toxin and involved in the pore formation and cell lysis (Li, *et al.*, 1991). There is a highly conserved hydrophobic sequence within the Domain 2 (Fig. 5.14) with 60% amino acid identity among all crystal proteins (Pereyra-Alférez, *et al.*, 1992). Therefore a similar hydrophobic sequence present in the $AnfA_1$ protein, might determine a toxin-like activity on the gut cells of the *C. zealandica* larvae.

A tentative promoter region was found about 300 bases from the first *Hind*III site in the Fig. 5.11. The location of this promoter is consistent with the previously proposed promoter P1 (see section 3.3.2 and Fig. 5.5). Thus, the transcription of the hybrid ß-galactosidase observed in the mutant pMMU48 (Fig. 5.4) might have been initiated in this promoter region, since the mini-Mu insertion was mapped around 300 bp from the *Bam*HI restriction site (see Fig. 5.5). It was suggested that the P1 promoter could collaborate in the expression of AnfA and AnfB proteins (section 4.1). However, palindrome[6] sequences that might function as a transcription terminator were found at position 440 and 468 (CGGCACTAGCCATTGTCATGCTGGTGCCG), that is, upstream the *anfA* and *anfB* genes. Therefore, the influence of the P1 promoter on the expression of both proteins is unclear.

In summary the *amb2* locus causing anti-feeding effect encodes two main proteins named AnfA and AnfB which seem to be essential for the AFE phenotype. The protein $AnfA_2$, deduced from DNA sequencing data, might also collaborate in the AFE phenotype conforming a multimeric molecule in connection with $AnfA_1$ and/or AnfB proteins. Further analysis of the *amb2* locus to define the organization and regulation of the involved genes, is essential to elucidate the precise role of the Anf proteins in amber disease.

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[6] Palindrome is a sequence of DNA that is the same when one strand is read left to right or the other is read right to left: consist of adjacent inverted repeats (Lewin, 1987).

CHAPTER VI

FINAL DISCUSSION AND CONCLUSION

Bacterial pathogenicity depends on a complex series of events and requires multiple virulence factors (see Chapter I). In this work, three virulence determinants associated with amber disease caused by *S. entomophila* to larvae of *C. zealandica* have been identified and analyzed: i) MRE-HA fimbriae; ii) the extracelluar protease of *S. entomophila* and iii) the anti-feeding activity caused by the *amb2* locus. The results of this research cover the general and the specific aims defined in Chapter I.

The three factors studied, the fimbriae, the extracellular protease and the anti-feeding activity seem to work in collaboration to determine the development of amber disease. Other unknown factors very likely are also involved in the disease. However, the fact that amber disease symptoms produced by *S. entomophila* are mimicked by supernatants from pathogenic strains (Chapter IV) indicates that an extracellular toxin is essential for the development of amber disease. This is consistent with the appearance of anti-feeding effect and amber coloration before the bacteria had invaded the insect haemocoel, observation that originally suggested the involvement of an anti-feeding toxin in amber disease (Jackson, *et al.*, 1993). Inhibition of trypsin production by the midgut cells in larvae suffering amber disease has been reported (Glare, *et al.*, 1993b), which is in agreement with this hypothesis. Since bacterial colonization occurs mainly on the foregut of the larvae and only few *S. entomophila* cells have been found outside this region (Glare, *et al.*, 1993b) it is reasonable to think that an extracellular *S. entomophila* toxin may be implicated.

One of the closest relatives to *S. entomophila* is *S. marcescens* as was demonstrated by the percentage of homology between both DNA genomes (Grimont, *et al.*, 1988). However, the pathogenic determinants associated with their infections seem to be different. Visible symptoms of disease caused by *S. marcescens* have not been reported and the final insect death has been characterized by invasion of the haemocoel. Variability of results observed in bioassays with *S. marcescens* even in similar conditions and with the same bacteria strains, has suggested *Serratia* spp. are opportunist pathogens which multiply rapidly if they gain access to the haemocoel of stressed insects hosts (Steinhaus, 1959; Podgwaite and Cosenza, 1976b). *S. entomophila* causing amber disease appears to share some of these features as there is also invasion of the haemocoel at the end of the disease (Jackson, *et al.*, 1993).

Nevertheless, the hypothesis on the participation of a toxin strongly supported by the evidence presented here, offers new alternatives in the understanding of amber disease and *Serratia* infections.

The data suggest that cessation of feeding displayed by larvae fed on *S. entomophila* mutants carrying *amb2* locus is caused by proteins with toxic activity on *C. zealandica* (Chapter 4). It was demonstrated (Chapter V) that *amb2* locus encodes at least three polypeptides involved in amber disease: $AnfA_1$, $AnfA_2$ and AnfB. Therefore, these proteins might be subunits of a multimeric toxin. Bacterial toxins are usually composed of two discrete subunits or domains: the subunit or domain A contains a specific enzymatic function and the subunit B confers the binding domain that interacts with a cell membrane receptor (see Chapter I). The subunit B may be an oligomer formed with several molecules which may be similar, as it was observed in cholera toxin (Spangler, 1992), or different, as in pertussis toxin (Tamura, *et al.*, 1982). Then, the $AnfA_2$ protein might function in connection with $AnfA_1$ and/or AnfB protein. The synthesis of other polypeptides by the *amb2* locus, not detected in this work, is not excluded. The 1.3 *Hind*III fragment dividing the *anfA* and *anfB* genes (see Fig. 5.3) might contains potential sequences coding for other proteins.

Potential regulatory sequences were also observed in the promoter region upstream the translation initiation codon of both $AnfA_1$ and $AnfA_2$ polypeptides. These sequences are associated with regulation of virulence genes as the ToxR-binding site in *V. cholerae* and the Fur-binding site in *E. coli* (Schaffer, *et al.*, 1985; Miller, *et al.*, 1987), which supports the hypothesis that both proteins are virulence factors.

Another fact supporting the role of the AnfA and AnfB proteins as a toxin is that some homology was observed between a hydrophobic region of the domain 2 of the δ-endotoxin of *B. thuringiensis*, which is essential for the entomocidal activity, and the $AnfA_1$ protein (Chapter V). In fact, the external symptom of intoxication shown by insects larvae fed with the δ-endotoxin of *B. thuringiensis* is cessation of feeding (Aronson, *et al.*, 1986). Then, the anti-feeding effect caused by the *amb2* gene products might be the result of paralysis of the gut and changes of the gut permeability, as was observed in larvae fed with the δ-endotoxin (Aronson, *et al.*, 1986). *C. zealandica* larvae suffering from amber disease stop feeding and also stop voiding faeces from the digestive tract, which might be caused by paralysis of the gut. Inhibition of trypsin production by the midgut cells observed in amber diseased

larvae (Glare, *et al.*, 1993b), might be also associated with gut paralysis. Even though only low homology was found between the *anfA* gene and the δ-endotoxins of *B. thuringiensis*, similarity between the three-dimensional structure of the active domains of both proteins may implay some form of membrane insertion and might render similar toxic activity.

Three types of fimbriae (type 1, type 3 and MRE-HA fimbriae) were identified and characterized in the wild type pathogenic strains of *S. entomophila.* Analysis of nonpathogenic *S. entomophila* mutants suggested that the MRE-HA fimbriae was the one associated with pathogenicity.

The extracellular protease of *S. entomophila* was identified and cloned. Examination and complementation assays of pathogenic and nonpathogenic strains, showed that the protease is not directly involved in amber disease but it might play and indirect role to potentiate the disease. It was also suggested that the extracellular protease might be linked to pathogenicity by means of a common regulatory factor.

Based on these results a model of amber disease is proposed. Proteins encoded by the *amb2* locus could function as specific toxin for the larvae causing cessation of feeding and clearing of the gut resulting in the development of amber coloration. Bacteria adhesion to the foregut mediated by the MRE-HA fimbriae might be important for colonization and further multiplication of the bacteria. Proteases and chitinases activity would lead at the end of the disease to invasion of the haemocoel by the pathogen and death of the insect. Whether the first events in amber disease are adhesion and colonization is not clear, since cessation of feeding occurs at a stage when there are very few adhering bacteria in the foregut (Jackson, *et al.*, 1993). Therefore the anti-feeding toxin seems to be the primary factor determining amber disease. The expression of the anti-feeding toxin might be enhanced after adhesion and multiplication of the bacteria. Proteases might potentiate the disease by attacking the insect immune system and/or processing the anti-feeding toxin. On the other hand, since adhesion and consequent colonization of *S. entomophila* occurs mainly on the foregut, the extracellular protease, the chitinases and the anti-feeding toxin would be spread out by the gut contents along the digestive tract. These three proteins would act in collaboration to produce disintegration of the foregut and midgut membranes. Finally the bacteria would invade the haemocoel and produce septicemia.

The findings presented here offer new potentialities for insect biological control by *Serratia* species. This is the first evidence on a possible toxin causing insect cessation of feeding expressed by *Serratia* spp. In addition this is also the first report on the isolation of a genetic region encoding proteins associated with a toxin-like activity on a member of the Scarabaeidae. Several other non-sporeforming bacteria such as *Pseudomonas aeruginosa*, *S. marcescens* and *Micrococcus nigrofasciens* have been reported as potential pathogens for scarabs (Poprawski and Yule, 1990) but the character of their infections seems to be invasive. Sporeforming bacteria as *Bacillus popillia* and *Bacillus lentimorbus* have been shown responsible of milky disease to scarabs (Dutky, 1940; Tashiro and Steinkraus, 1966). However, due to the inability to culture the cells *in vitro*, the use of the bacteria as an extensive biological control has been limited. *Bacillus cereus* has been reported also attacking scarabs and even though it grows readily *in vitro*, it needs further evaluation as a potential biological control agent (Poprawski and Yule, 1990). New strains of *B. thuringiensis* found to be toxic for *C. zealandica* (Wigley and Chilcott, 1990) also require further evaluation.

As mentioned previously, field tests with *S. entomophila* have shown high levels of pathogenicity to *C. zealandica* (Jackson, *et al.*, 1986). The bacteria has been developed as a commercial bioinsecticide and recently it has been introduced onto the New Zealand market (Jackson, *et al.*, 1992). Although the bacterium is highly specific, it provides a potentially powerful strategy for searching and developing new *Serratia* spp. strains with wider scarab insecticidal spectra.

The molecular genetics approach used to identify and isolate the genetic locus is relevant as it provides a direct way for finding out the pathogenic determinants of the disease at the level of DNA. It offers great potentialities for application of gene transfer programs as well as on studies for characterizing the factors involved in the disease in order to improve biological control systems.

Future research on this project might be aimed to isolate and characterize the anti-feeding toxin encoded by the amb2 locus. Sequencing of the *anfB* gene would offer valuable information to a better understanding of the toxin and its regulation.

CONCLUSION

It is concluded that amber disease caused by *S. entomophila* to the grass grub

larvae *C. zealandica* depend on multiple virulence factors. Genetic evidence was presented in this work showing that the gene products of the *amb2* locus are responsible for the anti-feeding effect (AFE) observed in infected larvae. Other factors as MRE-HA fimbriae and the extracellular protease of *S. entomophila* act in collaboration to potentiate the disease. Further analysis of the *amb2* locus and the isolation of the Anf proteins are required to define its precise role in pathogenicity.

This study provides an integrationist approach to understand the molecular basis of amber disease and it is in agreement with recent conclusions obtained by Dorman and Bhriain (1992) about pathogenic bacteria:

"the reductionist philosophy which has served biology so well in the past is now giving way to an integrationist approach in which it is possible to link apparently discrete pieces of information in order to provide a unified picture of the system of interest".

It is becoming evident that virulence genes function in collaboration to determine pathogenicity and also, that they are linked by a network of regulatory factors. Therefore, as it was stated in Chapter I, the study of bacterial pathogenicity requires an integral analysis between the pathogenic determinants and their regulatory systems.

REFERENCES

1. **Actis, L. A., Potter, S. A., and Crosa, J. H.** (1985). Iron-regulated outer membrane protein OM2 of *Vibrio anguillarum* is encoded by virulence plasmid pJM1. J. Bacteriol. *161*, 736-742.

2. **Adegbola, R. A., and Old, D. C.** (1982). New fimbrial hemmagglutinin in *Serratia* species. Infect. Immun. *38*, 306-315.

3. **Adegbola, R. A., Old, D. C., and Aleksic, S.** (1983). Rare MR/K-like hemagglutinins (and type 3-like fimbriae of *Salmonella* strains. FEMS Microbiol. Lett. *19*, 233-238.

4. **Adler, H. I., Fisher, W. D., Cohen, A., and Hardigree, A. A.** (1967). Miniature *Escherichia coli* cells deficient in DNA. Proc. Natl. Acad. Sci. USA *57*, 321-326.

5. **Albright, L. M., Huala, E., and Ausubel, F. M.** (1989). Prokaryotic signal transduction mediated by sensor and regulator protein pairs. Annu. Rev. Genet. *23*, 311-336.

6. **Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J.** (1990). Basic local alignment search tool. J. Mol. Biol. *215*, 403-410.

7. **Aronson, A. I., Beckman, W., and Dunn, P.** (1986). *Bacillus thuringiensis* and related insect pathogens. Microbiol. Rev. *50*, 1-24.

8. **Aronson, M., Medalia, D., Schori, L., Mirelman, D., Sharon, N., and Ofek, I.** (1979). Prevention of colonization of the urinary tract of mice with *Escherichia coli* by blocking of bacterial adherence with methyl-D-mannophyranoside. J. Infect. Dis. *139*, 322-329.

9. **Bachmann, B. J.** (1990). Linkage map of *Escherichia coli* K-12, edition 8. Microbiol. Rev. *54*, 130-197.

10. **Beringer, J. E., Benyon, J. L., Buchanan-Wollston, A. V., and Johnston, A. W. V.** (1977). The isolation of conditional ineffective mutants of *Rhizobium leguminosarum*. J. Gen. Microbiol. *98*, 339-343.

11. **Bernardini, M. L., Fontaine, A., and Sansonetti, P. J.** (1990). The two-component regulatory system OmpR-EnvZ controls the virulence of *Shigella flexneri*. J. Bacteriol. *172*, 6274-6281.

12. **Betley, M. J., Miller, V. L., and Mekalanos, J. J.** (1986). Genetics of bacterial enterotoxins. Ann. Rev. Microbiol. *40*, 577-605.

13. **Bever, R. A., and Iglewski, B. H.** (1988). Molecular characterization and nucleotide sequence of the *Pseudomonas aeruginosa* elastase structural gene. J. Bacteriol. *170*, 4309-4314.

14. **Blomfield, I. C., McClain, M. S., and Eisenstein, B. I.** (1991). Type 1 fimbriae mutants of *Escherichia coli* K12:characterization of recognized afimbriate strains and construction of new *fim* deletion mutants. Mol. Microbiol. *5*, 1439-1445.

15. **Bolivar, F., Rodriguez, R. L., Betlach, M. C. and Boyer, H. W.** (1977). Construction and characterization of new cloning vehicles. I. Ampicillin resistant

derivatives of the plasmid pMB9. Gene 2, 75-93.

16. Booth, B. A., Finkelstein, M. B., and Finkelstein, R. A. (1984). *Vibrio cholerae* hemagglutinin/protease nicks cholera enterotoxin. Infect. Immun. *45*, 558-560.

17. Booth, B. A., Boesman-Finkelstein, M., and Finkelstein, R. A. (1983). *Vibrio cholerae* soluble hemagglutinin/protease is a metalloenzyme. Infect. Immun. *42*, 639-644.

18. Bottonley, W. (1987) (unpublished)

19. Botsford, J. L., and Harman, J. G. (1992). Cyclic AMP in prokaryotes. Microbiol. Rev. *56*, 100-122.

20. Boyd, J., Oso, M. N., and Murphy, J. R. (1990). Molecular cloning and DNA sequence analysis of a diphtheria *tox* iron-dependent regulatory element *dtxR* from *Corynebacterium diphtheriae*. Proc. Natl. Acad. Sci. USA *87*, 5968-5972.

21. Bromke, B. J., and Hammel, J. M. (1979). Regulation of extracellular protease formation by *Serratia marcescens*. Can. J. Microbiol. *25*, 47-52.

22. Bromke, B. J., and Hammel, J. M. (1978). Regulation of extracellular protease formation by *Serratia marcescens*. Can. J. Microbiol. *25*, 47-52.

23. Bucher, G. E. (1960). Potential pathogens of insects and their characteristics. J. Insect Pathol. *2*, 172-195.

24. Burrows, M. R., Sellwood, R., and Gibbons, R. A. (1976). Haemagglutinating and ahesive properties associated with the K99 antigen of bovine strains of *Escherichia coli*. J. Gen. Microbiol. *96*, 269-275.

25. Calderwood, S. B., and Mekalanos, J. J. (1988). Confirmation of the Fur operator site by insertion of a synthetic oligonucleotide into an operon fusion plasmid. J. Bacteriol. *170*, 1015-1017.

26. Calderwood, S. B., and Mekalanos, J. J. (1987). Iron regulation of Shiga-like toxin expression in *Escherichia coli* is mediated by the *fur* locus. J. Bacteriol. *169*, 4759-4764.

27. Cameron, D. J., and Wigley, P. G. (1989). *Costelytra zealandica* (White) grass grub (Coleoptera:Scarabaeidae). In A review of biological control of invertebrate pest and weed in New Zealand, 1974 to 1987, P. J. Cameron, R. L. Hill, J. Bain, and W. P. Thomas, eds. (CAB International), pp. 9-16.

28. Casadaban, M. J., and Chou, J. (1984). *In vivo* formation of gene fusions encoding hybrid β-galactosidase proteins in one step with a transposable Mu-*lac* transducing phage. Proc. Natl. Acad. Sci. USA *81*, 535-539.

29. Casadaban, M. J., Chou, J., and Cohen, S. N. (1980). In vitro gene fusions that join an enzymatically active β-galactosidase segment to amino-terminal fragments of exogenous proteins: *Escherichia coli* plasmid vectors for the detection and cloning of translational initiation signals. J. Bacteriol. *143*, 971-980.

30. Cassel, D., and Pfeuffer, T. (1978). Mechanism of cholera toxin action: covalent modification of the guanyl nucleotide-binding protein of the adenylate cyclase

system. Proc. Natl. Acad. Sci. USA *75*, 2669-2673.

31. Castilho, B. A., Olfson, P., and Casadaban, M. J. (1984). Plasmid insertion mutagenesis and *lac* gene fusion with Mini-Mu bacteriophage transposons. J. Bacteriol. *158*, 488-495.

32. Chadwick, J. S., Caldweel, S. S., and Chadwick, P. (1990). Adherence patterns and virulence for *Galleria mellonella* larvae of isolates of *Serratia marcescens*. J. Invertebr. Pathol. *55*, 133-134.

33. Chambers, J. A., Jelen, A., Gilbert, M. P., Dany, C. S., Johnson, T. B., and Gawron-Burke, C. (1991). Isolation and characterization of a novel insecticidal crystal protein gene from *Bacillus thuringiensis* subsp. *aizawai*. J. Bacteriol. *173*, 3966-3976.

34. Chapman, R. B. (1990). Insect pest. In Pastures: their ecology and management, R. H. M. Langer, ed. (Oxford: Oxford University Press), pp. 448-467.

35. Chatfield, S. N., Dorman, C. J., Hayward, C., and Dougan, G. (1991). Role of *ompR*-dependent genes in *Salmonella thyphymurium* virulence mutants deficient in both OmpC and OmpF are attenuated *in vivo*. Infect. Immun. *59*, 449-454.

36. Chowdhury, M. A. R., Miyoshi, S. I., and Shinoda, S. (1990). Purification and characterization of a protease produced by *Vibrio mimicus*. Infect. Immun. *58*, 4159-4162.

37. Clegg, S., and Gerlach, G. F. (1987). Enterobacterial fimbriae. J. Bacteriol. *169*, 934-938.

38. Collier, R. J. (1975). Diphtheria toxin mode of action and structure. Bacteriol. Rev. *39*, 54-85.

39. Comai, L., and Kosuge, T. (1982). Cloning and characterization of *iaaM*, a virulence determinant of *Pseudomonas savastanoi*. J. Bacteriol. *149*, 40-46.

40. Corbett, G. E. (1990). Analysis of chitinolytic loci in *Serratia entomophila*, a pathogen of the New Zealand grass grub (*Costelytra zealandica* [White]) [Masters of Science Thesis]. (University of Canterbury, Christchurch, New Zealand).

41. Crowther, R. S., Roomi, N. W., Fahim, R. E. F., and Forstner, J. F. (1987). *Vibrio cholerae* metalloprotease degrades intestinal mucin and facilitates enterotoxin-induced secretion from rat intestine. Biochim. Biophys. Acta *924*, 393-402.

42. Curtiss, R., and Kelly, S. M. (1987). *Salmonella typhimurium* deletion mutants lacking adenylate cyclase and cyclic AMP receptor protein are avirulent and immunogenic. Infect. Immun. *55*, 3035-3043.

43. Dahler, G. S., Barras, F., and Keen, N. T. (1990). Cloning of genes encoding estracellular metalloproteases from *Erwinia chrysanthemi* EC16. J. Bacteriol. *172*, 5803-5815.

44. Dearing, S. C., Scotti, P. D., Wigley, P. J., and Ohana, S. D. (1980). A small RNA virus from the grass grub *Costelytra zealandica* (Coleoptera:Scarabaeidae). New Zealand J. Zool. *7*, 267-269.

45. De Lorenzo, V., Herrero, M., Giovannini, F., and Neilands, J. B. (1988). Fur (ferric uptake regulon) protein and CAP (catabolite-activator protein) modulate transcription of *fur* gene in *Escherichia coli*. Eur. J. Biochem. *173*, 537-546.

46. DeVault, J. D., Berry, A., Misra, T. K., Darzins, A., and Chakrabarty, A. M. (1989). Environmental sensory signals in microbial pathogenesis:*Pseudomonas aeruginosa* infection in cystic fibrosis. Biotech. *7*, 352-357.

47. DiRita, V. J. (1992). Co-ordinate expression of virulence genes by ToxR in *Vibrio cholerae*. Mol. Microbiol. *6*, 451-458.

48. DiRita, V. J., and Mekalanos, J. J. (1989). Regulation of bacterial virulence. Annu. Rev. Genet. *23*, 455-482.

49. DiRita, V. J., Parsot, C., Jander, G., and Mekalanos, J. J. (1992). A regulatory cascade controls virulence in *Vibrio cholerae*. In Microbial Adhesion and invasion, M. Hook, and L. Switalski, eds. (New York: Springer-Verlag), pp. 77-83.

50. DiRita, V. J., Parsot, C., Jander, G., and Mekalanos, J. J. (1991). Regulatory cascade controls virulence in *Vibrio cholerae*. Proc. Natl. Acad. Sci. USA *88*, 5403-5407.

51. Ditta, G., Stanfield, S., Corbin, D., and Helinski, D. R. (1980). Broad host range DNA cloning system for gram-negative bacteria:construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA *77*, 7347-7351.

52. Dorman, C. J., and Bhriain, N. N. (1992). Global regulation of gene expression during environmental adaptation:implications for bacterial pathogens. In Molecular Biology of Bacterial Infection, C. E. Hormaeche, C. W. Penn, and C. J. Smyth, eds. (Cambridge: Cambridge University Press), pp. 193-230.

53. Dorman, C. J., Chatfields, S., Higgins, C. F., Hayward, C., and Dougan, G. (1989). Characterization of porin and *ompR* mutants of a virulent strain of *Salmonella typhimurium*:*ompR* mutants are attenuated *in vivo*. Infect. Immun. *57*, 2136-2140.

54. Duguid, J. P. (1959). Fimbriae and adhesive properties in *Klebsiella* strains. J. Gen. Microbiol. *21*, 271-286.

55. Duguid, J. P., Clegg, S., and Wilson, M. I. (1979). The fimbrial and non-fimbrial haemagglutinins of *Escherichia coli*. J. Med. Microbiol. *12*, 213-227.

56. Dutky, S. R. (1940). Two new spore-forming bacteria causing milky disease of the japanese beetle. J. Ag. Res. *61*, 57-68.

57. Eisenstein, B. I., and Dodd, D. C. (1982). Pseudocatabolite repression of type 1 fimbriae of *Escherichia coli*. J. Bacteriol. *151*, 1560-1567.

58. Eisenstein, B. I., Beashey, E. H., and Solomon, S. S. (1981). Divergent effects of cyclic adenosine 3', 5'-monophosphate on formation of type 1 fimbriae in different K-12 strains of *Escherichia coli*. J. Bacteriol. *145*, 620-623.

59. Elwell, L. P., and Shipley, P. L. (1980). Plasmid-mediated factors associated with virulence of bacteria to animals. Ann. Rev. Microbiol. *34*, 465-496.

60. Evans, D. G., and Evans, D. J. (1978). New surface-associated heat-labile colonization factor antigen (CFA/II) produced by enterotoxigenic *Escherichia coli* of serogroups 06 and 08. Infect. Immun. *21*, 638-647.

61. Evans, D. G., Evans, D. J., Clegg, S., and Pauley, J. (1979). Purification and characterization of CFA/I antigen of enterotoxigenic *Escherichia coli*. Infect. Immun. *25*, 738-748.

62. Evans, D. G., Evans, D. J., Tjoa, W. S., and DuPont, H. L. (1978). Detection and characterization of the colonization factor of enterotoxigeneic *Escherichia coli* isolated from adults with diarrhea. Infect. Immun. *19*, 727-736.

63. Evans, D. G., Evans, D. J., and Tjoa, W. (1977). Hemagglutination of human group A erythrocytes by enterotoxigenic *Escherichia coli* isolated from adults with diarrhea: correlation with colonization factor. Infect. Immun. *18*, 330-337.

64. Fader, R. C., and Davis, C. P. (1980). Effect of piliation on *Klebsiella pneumoniae* infection in rat bladders. Infect. Immun. *30*, 554-561.

65. Finlay, B. B. (1992). Molecular genetic approaches to understanding bacterial pathogenesis. In Molecular biology of bacterial infection. Current status and future perspectives, C. E. Hormaeche, C. W. Penn, and C. J. Smyth, eds. (Cambridge: Cambridge University Press), pp. 33-45.

66. Finlay, B. B., and Falkow, S. (1989). Common themes in microbial pathogenicity. Microbiol. Rev. *52*, 210-230.

67. Flyg, C., and Boman, H. G. (1988). *Drosophila* genes cut and miniature are associated with susceptibility to infection by *Serratia marcescens*. Gen. Res. *52*, 51-56.

68. Flyg, C., and Xanthopoulos, K. G. (1983). Insect pathogenic properties of *Serratia marcescens*. Passive and active resistance to insect immunity studied with protease-deficient and phage resistant mutants. J. Gen. Microbiol. *129*, 453-464.

69. Forsman, K., Sonden, B., Goransson, M., and Uhlin, B. E. (1992). Antirepression function in *Escherichia coli* for the cAMP-CRP receptor protein transcriptional activator. Proc. Natl. Acad. Sci. USA *89*, 9880-9884.

70. Fowler, M. (1974). Milky disease (*Bacillus* spp.) occurrence and experimental infection of *Costelytra zealandica* and other Scarabaeidae. New Zealand J. Zool. *1*, 97-109.

71. Fuchs, R. L., McPherson, S. A., and Drahos, D. J. (1986). Cloning of a *Serratia marcescens* gene encoding chitinase. App. Environ. Microbiol. *51*, 504-509.

72. Gilbert, I., Villegas, V., and Barbe, J. (1989). Expression of heat labile enterotoxin is under cyclic AMP control in *Escherichia coli*. Curr. Microbiol. *20*, 83-90.

73. Gill, D. M. (1976). The arrangement of subunits in cholera toxin. Biochem. *15*, 1242-1248.

74. Gill, D. M., and Meren, R. (1978). ADP-ribosylation of membrane proteins catalyzed by cholera toxin: basis of the activation of adenylate cyclase. Proc. Natl.

Acad. Sci. USA *75*, 3050-3054.

75. Glare, T. R., and Jackson, T. A. (1990). The infection process of the New Zealand grass grub, *Costelytra zealandica*, by the bacterium *Serratia entomophila*. Abstract for the Vth International Colloquium on Invertebrate Pathology and Microbial Control, Adelaide, Australia, 20-24 August.

76. Glare, T. R., Corbett, G. E., and Sadler, T. J. (1993). Association of a large plasmid with Amber disease of the New Zealand grass grub, *Costelytra zealandica* caused by *Serratia entomophila* and *Serratia proteamaculans*. J. Invertebr. Pathol. *62*, 165-170.

77. Glare, T. R., Jackson, T. A., and Binnington, K. C. (1993b). Growth of *Serratia entomophila* (Enterobacteriaceae), causing amber disease, in the gut of the New Zealand grass grub (*Costelytra zealandica*). 6th Australasian Conference on Grassland Invertebrate Ecology, 17-19 February, Hamilton, New Zealand.

78. Glare, T. R., Upadhyaya, N. M., and Mahanty, H. K. (1992). Genetic manipulation towards strain improvement of scarab pathogens. In Use of pathogens in scarab pest management, 1st ed., T. A. Jackson, and T. R. Glare, eds. (Andover: Intercept Ltd.), pp. 153-165.

79. Göranson, M., Formsman, K., Nilsson, P., and Uhlin, B. E. (1989). Upstream activating sequences that are shared by two divergently transcribed operons mediated cAMP-CRP regulation of pilus adhesion in *Escherichia coli*. Mol. Microbiol. *3*, 1661-1668.

80. Goldberg, M. B., DiRita, V. J., and Calderwood, S. B. (1990). Identification of an iron-regulated virulence determinant in *Vibrio cholerae*, using Tn*phoA* mutagenesis. Infect. Immun. *58*, 55-60.

81. Grimont, P. A. D., Grimont, F., and Lysenko, O. (1979). Species and biotype identification of *Serratia* strains associated with insects. Curr. Microbiol. *2*, 139-142.

82. Grimont, P. A. D., Grimont, F., and Dulong, H. L. C. (1977). Characterization of *Serratia marcescens, S. liquefaciens, S. plymuthica* and *S. marinorubra* by electrophoresis of their proteinases. J. Gen. Microbiol. *99*, 301-310.

83. Grimont, P. P. D., Jackson, T. A., Ageron, E. E., and Noonan, M. J. (1988). *Serratia entomophila* sp. nov, a species associated with amber disease in the New Zealand grass grub, *Costelytra zealandica*. Int. J. Syst. Bact. *38*, 1-6.

84. Hacker, J. (1990). Genetic determinants coding for fimbriae and adhesins in extraintestinal *Escherichia coli*. Curr. Top. Microbiol. Immun. *15*, 1-27.

85. Häse, C. C., and Finkelstein, R. A. (1991). Cloning and nucleotide sequence of the *Vibrio cholerae* hemagglutinin/protease (HA/Protease) gene and construction of an HA/Protease-negative strain. J. Bacteriol. *173*, 3311-3317.

86. Hagberg, L., Jodal, U., Korhonen, T. K., Lidin-Johnson, G., Linberg, U., and Svanborg-Eden, C. (1981). Adhesion, hemagglutination and virulence of *Escherichia coli* causing urinary tract infections. Infect. Immun. *31*, 564-570.

87. Haider, M. Z., Knowles, B. H., and Ellar, D. J. (1986). Specificity of *Bacillus thuringiensis* var *colmeri* insecticidal delta-endotoxin by differential processing of the

protoxin by larval gut proteases. Eur. J. Biochem. *156*, 531-540.

88. Heussen, C., and Dowdle, E. B. (1980). Electrophoretic analysis of plasminogen activators in polyacrylamide gels contining sodium dodecyl sulfate and copolymerized substrates. Anal. Biochem. *102*, 196-202.

89. Hines, D. A., Saurugger, P. N., Ihler, G. M., and Benedik, M. J. (1988). Genetic analysis of extracellular proteins of *Serratia marcescens*. J. Bacteriol. *170*, 4141-4146.

90. Höfte, H., and Whiteley, H. R. (1989). Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol. Rev. *53*, 242-255.

91. Höfte, H., Soetaert, P., Jansens, S., and Peferoen, M. (1990). Nucleotide sequence and deduced aminoacid sequence of a new lepidopteran-specific crystal protein gene from *Bacillus thuringiensis*. Nucl. Acids. Res. *18*, 5545.

92. Holder, I. A., and Haidaris, C. G. (1979). Experimental studies of the pathogenesis of infections due to *Pseudomonas aeruginosa* extracellular protease and elastase as *in vivo* virulence factor. Can. J. Microbiol. *25*, 1979.

93. Hoschützky, H., Lottspeich, F., and Jann, K. (1989). Isolation and characterization of the α-galactosy;-1,4-β-galactosyl-specific adhesin (P adhesin) from fimbriated *Escherichia coli*. Infect. Immun. *57*, 76-81.

94. Hoy, J. M. (1965). The influence of insects on pasture production. N.Z. Agric. Sci. *1*, 21-24.

95. Hultgren, S. J., Lindberg, F., Magnusson, G., Kihlberg, J., Tennent, J. M., and Normark, S. (1989). The PapG adhesin of uropathogenic *Escherichia coli* contains separate regions for receptor binding and for the incorporation into pilus. Proc. Natl. Acad. Sci. USA *86*, 4357-4361.

96. Hultgren, S. J., Porter, A. J., Schaeffer, A. J., and Duncan, J. L. (1985). Role of type 1 pili and effects of phase variation on lower urinary tract infections produced by *Escherichia coli*. Infect. Immun. *50*, 370-377.

97. Iglewski, B. H., and Kabat, D. (1975). NAD-dependent inhibition of protein synthesis by *Pseudomonas aeruginosa* toxin. Proc. Natl. Acad. Sci. USA *72*, 2284-2288.

98. Iglewski, B. H., Liu, P. V., and Kabat, D. (1977). Mechanism of action of *Pseudomonas aeruginosa* exotoxin A: adenosin diphosphate-ribosylation of mammalian elongation factor 2 *in vitro* and *in vivo*. Infect. Immun. *15*, 138-144.

99. Ip, S. M., Crichton, P. B., Old, D. C., and Duguid, J. P. (1981). Mannose-resistant and eluting haemagglutinins and fimbriae in *Escherichia coli*. J. Med. Microbiol. *14*, 223-226.

100. Jackson, T. A., Huger, A. M., and Glare, T. R. (1993). Pathology of amber disease in the New Zealand grass grub *Costelytra zealandica* (Coleoptera:Scarabaeidae). J. Invertebr. Pathol. *61*, 123-130.

101. Jackson, T. A., Pearson, J. F., and O'Callaghan, M. (1992). Pathogen to product - Development of *Serratia entomophila* (Enterobacteriacea) as a comercial biological control agent for the New Zealand grass grub (*Costelytra zealandica*). In

Use of Pathogens in Scarab Pest Management, T. A. Jackson, and T. R. Glare, eds. (Andover, Hampshire: Intercept), pp. 191-198.

102. Jackson, T. A., Pearson, J. F., and Stucki, G. (1986). Control of grass grub *Costelytra zealandica* (White) (Coleoptera:Scarabaeidae), by application of the bacteria *Serratia* spp. causing honey disease. Bull. Ent. Res. *76*, 69-76.

103. Jagusztyn-Krynicka, E. K., Smorawinska, M., and Curtiss, R. I. I. I. (1982). Expression of *Streptococcus mutants* aspartate-semialdehyde dehydrogenase gene c brad into pBR322. J. Gen. Microbiol. *120*, 1135-1145.

104. Jingushi, S., Mitsuyama, T., Morya, T., and Amako, K. (1987). Antigenic analysis of *Serratia marcescens* fimbriae with monoclonal antibodies. Infect. Immun. *55*, 1600-1606.

105. Jones, G. W., and Rutter, J. M. (1972). Role of the K88 antigen in pathogenesis of neonatal diarrhea caused by *Escherichia coli* in piglets. Infect. Immun. *6*, 918-927.

106. Karjalainen, T. K., Evans, D. G., Evans, D. J., Graham, D. Y., and Lee, C. H. (1991). Iron represses the expression of CFA/I fimbriae of enterotoxigenic *Escherichia coli*. Microbial Path. *11*, 317-323.

107. Kaska, M. (1976). The toxicity of extracellular proteases of the bacterium *Serratia marcescens* for larvae of greater wax moth *Galleria mellonella*. J. Invertebr. Pathol. *27*, 271.

108. Knowles, B. H., and Ellar, D. J. (1987). Colloid-osmotic lysis is a general feature of the mechanism of action of *Bacillus thuringiensis* δ-endotoxins with different insect specificities. Biochim. Biophys. Acta *924*, 509-518.

109. Kohno, K., Yamamoto, T., Kutoiwa, A., and Amako, K. (1984). Purification and characterization of *Serratia marcescens* US5 pili. Infect. Immun. *46*, 295-300.

110. Korhonen, T. K., Vaisanen, R. V., Rhen, M., Pere, A., Parkkinen, J., and Finne, J. (1984). *Escherichia coli* fimbriae recognizing sialyl-galactosides. J. Bacteriol. *159*, 762-766.

111. Korhonen, T. K., Vaisanan, V., Kallio, P., Nurmiako-Lassila, E. L., Ranta, H., Shtonen, A., Eol, J., Svenson, S. B., and Svanborg-Eden, C. (1982a). The role of pili in the adhesion of *Escherichia coli* to human urinary tract epithelial cells. Scand. J. Infect. Dis. *33 (Suppl.)*, 26-31.

112. Korhonen, T. K., Vaisanen, V., Saxen, H., Hultberg, H., and Svenson, S. B. (1982b). P-antigen-recognizing fimbriae from human uropathogenic *Escherichia coli* strains. Infect. Immun. *37*, 286-291.

113. Korhonen, T. K., Lounatmaa, K., Ranta, H., and Kuusi, N. (1980). Characterization of type 1 pili of *Salmonella typhimurium LT12*. J. Bacteriol. *144*, 800-805.

114. Kucera, M., and Lysenko, O. (1977). The mechanism of pathogenicity of *Pseudomonas aeruginosa*: milk-clotting activity of the proteolytic enzymes toxic for *Galleria mellonella* larvae. J. Invertebr. Pathol. *29*, 388-389.

115. Kutsukake, K., Ohya, Y., and Iino, T. (1990). Transcriptional analysis of the

flagellar region of *Salmonella typhimurium*. J. Bacteriol. *172*, 741-747.

116. Labigne-Roussel, A., Schmidt, M. A., Walz, W., and Falkow, S. (1985). Genetic organization of the afimbrial adhesin operon and nucleotide sequence from a uropathogenic *Escherichia coli* gene encoding an afimbrial adhesin. J. Bacteriol. *162*, 1285-1292.

117. Laemmli, U. K. (1970). Cleavage of structural protein during the assembly of the head of bacteriophage T4. Nature *227*, 680-685.

118. Lecadet, M. M., and Dedonder, R. (1967). Enzymatic hydrolysis of the crystals of *Bacillus thuringiensis* by the proteases of *Pieris brassicar*.I. Preparation and fractionation of the lysates. J. Invertebr. Pathol. *9*, 310-321.

119. Lerecleus, D. C., Bourgouin, M. M., Lecadet, M. M., Klier, A., and Rapoport, G. (1989). Role, structure and molecular organization of the genes coding for the parasporal δ-endotoxins of *Bacillus thuringiensis*. In Regulation of procaryotic development, I. Smith, R. A. Slepecki, and P. Setlow, eds. (Washington: American Society for Microbiology), pp. 255-276.

120. Leung, K. Y., and Stevenson, R. M. W. (1988). Tn5-induced protease-deficient strains of *Aeromonas hydrophila* with reduced virulence for fish. Infect. Immun. *56*, 2639-2644.

121. Lewin, B. (1987). Genes, 3rd ed. (New York: John Wiley & Sons).

122. Li, J., Carroll, J., and Ellar, D. J. (1991). Crystal structure of insecticidal δ-endotoxin from *Bacillus thuringiensis* at 2.5 Å resolution. Nature *353*, 815-821.

123. Lyerly, D. M., and Kreger, A. S. (1983). Importance of Serratia protease in the pathogenesis of experimental *Serratia marcescens* pneumonia. Infect. Immun. *40*, 113-119.

124. Lysenko, O. (1981). Principles of pathogenesis of insect bacterial diseases as exemplified by the nonsporeforming bacteria. In Pathogenesis of Invertebrate Microbial Diseases, E. W. Davidson, ed. (Totowa, N.J.: Allanheld, Osmun & Co. INC), pp. 163-188.

125. Lysenko, O. (1976). Chitinase of *Serratia marcescens* and its toxicity for insects. J. Invertebr. Pathol. *27*, 385-386.

126. Madziara-Borusiewicz, K., and Lysenko, O. (1971). The mechanisms of pathogenicity of *Pseudomonas aeruginosa* VII. The influence of toxic proteinase on haemocytes of *Galleria mellonella*. J. Invertebr. Pathol. *17*, 138-140.

127. Maniatis, T., Fritsch, E. F., and Sambrook, J., eds. (1982). Molecular cloning: a laboratory manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory).

128. Manning, G. (1993). Analysis of nonpathogenic mutants of *Serratia entomophila*. Third annual Queenstown molecular biology meeting. August 15-20, Queenstown, New Zealand).

129. Manoil, C., and Beckwith, J. (1985). Tn*phoA*: a transposon probe for protein export signals. Proc. Natl. Acad. Sci. USA *82*, 8129-8133.

130. Martinez-Cadena, M. G., Guzman-Verduzco, L. M., Steiglitz, H., and

Kuperstoch-Portnoy, Y. M. (1981). Catabolite repression of *Escherichia coli* heat-stable enterotoxin activity. J. Bacteriol. *145*, 722-728.

131. Mayan, M. C., Ofek, I., Medalia, O., and Aronson, M. (1985). Population shift in mannose-specific fimbriated phase of *Klebsiella pneumoniae* during experimental urinary tract infection in mice. Infect. Immun. *49*, 785-789.

132. McPheat, W. L., Wardlaw, A. C., and Novotny, P. (1983). Modulation of *Bordetella pertussis* by nicotinic acid. Infect. Immun. *41*, 516-522.

133. Melton, A. R., and Weiss, A. A. (1989). Environmental regulation of expression of virulence determinants in *Bordetella pertussis*. J. Bacteriol. *171*, 6206-6212.

134. Middlebrook, J. L., and Dorland, R. B. (1984). Bacterial toxins: cellular mechanisms of action. Microbiol. Rev. *48*, 199-221.

135. Miller, S. I., Kukral, A. M., and Mekalanos, J. J. (1989). A two-component regulatory system (*phoP phoQ*) controls *Salmonella typhimurium* virulence. Proc. Natl. Acad. Sci. USA *86*, 5054-5058.

136. Miller, V. L., and Mekalanos, J. J. (1988). A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. J. Bacteriol. *170*, 2575-2583.

137. Miller, V. L., and Mekalanos, J. J. (1985). Genetic analysis of the cholera toxin positive regulatory gene *toxR*. J. Bacteriol. *163*, 580-585.

138. Miller, V. L., Taylor, R. K., and Mekalanos, J. J. (1987). Cholera toxin transcriptional activator ToxR is a transmembrane DNA binding protein. Cell *48*, 271-279.

139. Mims, C. A. (1987). The pathogenesis of infectious disease. (London: Academic Press).

140. Minion, F. C., Abraham, S. N., Beachey, E. H., and Goguen, J. D. (1986). The genetic determination of adhesive function in type 1 fimbriae of *Escherichia coli* is distinct from the gene encoding the fimbrial subunit. J. Bacteriol. *165*, 1033-1036.

141. Miyata, K., Maejima, K., Tomoda, K., and Isono, M. (1970). *Serratia* protease. I. Purification and general properties of the enzyme. Agr. Biol. Chem. *34*, 310-318.

142. Mizunoe, y, Matsumoto, T., Amako, K., Sekiguchi, M., and Kumazawa, J. (1991). Identification and nucleotide sequence of the gene determining the adhesion capacity of *Serratia marcescens*. J. Bacteriol. *173*, 3257-3260.

143. Moore, S. G., Kalmakoff, J., and Miles, J. S. R. (1974). An iridescent virus and rickettsia from the grass grub *Costelytra zealandica* (Coleoptera:Scarabaeidae). New Zealand J. Zool. *1*, 205-210.

144. Nakahama, K., Yoshimura, K., Marumoto, R., Kikuchi, M., Lee, I. S., Hase, T., and Matsubara, H. (1986). Cloning and sequencing of *Serratia* protease gene. Nucl. Acids. Res. *14*, 5843-5855.

145. O'Hanley, P., Lark, D., Falkow, S., and Schoolnik, G. (1985). Molecular basis of *Escherichia coli* colonization of the upper urinary tract in BALB/c mice. J. Clin.

Invest. *75*, 347-360.

146. Old, D. C. (1985). Haemagglutination methods in the study of *Escherichia coli*. In The virulence of *Escherichia coli*, M. Sussman, ed. (London: Academic Press), pp. 287-313.

147. Old, D. C. (1972). Inhibition of the interaction between fimbrial haemagglutinin and erythrocytes by D-mannose and other carbohydrates. J. Gen. Microbiol. *71*, 149-157.

148. Old, D. C., and Adegbola, R. A. (1984). Relationships among broad-spectrum and narrow-spectrum mannose-resistant fimbrial haemagglutinins in different *Yersinia* species. Microbiol. Immunol. *28*, 1303-1311.

149. Old, D. C., and Duguid, J. P. (1970). Selective outgrowth of fimbriate bacteria in static liquid medium. J. Bacteriol. *103*, 447-456.

150. Old, D. C., and Payne, S. B. (1971). Antigens of the type 2 fimbriae of *Salmonellae*: "cross-reacting material" (CRM) of type 1 fimbriae. J. Med. Microbiol. *4*, 215-225.

151. Old, D. C., Tavaendale, A., and Senior, B. W. (1985). A comparative study of the type 3 fimbriae of *Klebsiella* species. J. Med. Microbiol. *20*, 203-214.

152. Old, D. C., Adegbola, R. A., and Scott, S. S. (1983). Multiple fimbrial haemagglutinins in *Serratia* species. Med. Microbiol. Immunol. *172*, 107-115.

153. Olsnes, S., Reisbig, R., and Eiklid, K. (1981). Subunit structure of *Shigella* cytotoxin. J. Biol. Chem. *256*, 8732-8738.

154. Otteman, K. M., DiRita, V. J., and Mekalanos, J. J. (1992). ToxR proteins with substitutions in residues conserved with OmpR fail to activate transcription from the cholera toxin promoter. J. Bacteriol. *174*, 6807-6814.

155. Paranchych, W., and Frost, L. S. (1988). The physiology and biochemistry of pili. Adv. Microbial Phys. *29*, 53-114.

156. Parment, P. A., Svanborg-Ede'n, C., Chaknis, M. J., Sawant, A. D., Hagberg, L., and Wilson, L. A. (1992). Hemagglutination (fimbriae) and hydrophobicity in adherence of *Serratia marcescens* to urinary tract epithelium and contact lenses. Curr. Microbiol. *25*, 113-118.

157. Parsot, C., and Mekalanos, J. J. (1990). Expression of ToxR, the transcriptional activator of the virulence factors in *Vibrio cholerae* is modulated by the heat shock response. Proc. Natl. Acad. Sci. USA *87*, 9898-9902.

158. Passador, L., Cook, J. M., Gambello, M. J., Rust, L., and Iglewski, B. H. (1993). Expression of *Pseudomonas aeruginosa* virulence genes requires cell to cell communication. Science *260*, 1127-1130.

159. Pearson, G. D. N., and Mekalanos, J. J. (1982). Molecular cloning of the *Vibrio cholerae* enterotoxin genes in *Escherichia coli* K12. Proc. Natl. Acad. Sci. USA *79*, 2976-2980.

160. Pereyra-Alférez, B., Bravo, A., Quintero, R., and Soberón, X. (1992). The δ-

endotoxin protein family displays a hydrophobic motif that might be implicated in toxicity. Mol. Microbiol. *6*, 2095-2098.

161. Peterson, K. M., and Mekalanos, J. J. (1988). Characterization of the Vibrio Cholerae ToxR regulon: Identification of novel genes involved in intestinal colonization. Infect. Immun. *56*, 2822-2929.

162. Podgwaite, J. D., and Cosenza, B. J. (1976a). A strain of *Serratia marcescens* pathogenic for larvae of *Lymantria dispar*: characterization. J. Invertebr. Pathol. *27*, 185-190.

163. Podgwaite, J. D., and Cosenza, B. J. (1976b). A strain of *Serratia marcescens* pathogenic for larvae of *Lymantria dispar*: Infectivity and mechanisms of pathogenicity. J. Invertebr. Pathol. *27*, 199-208.

164. Poole, K., and Braun, V. (1988). Iron regulation of *Serratia marcescens* hemolysin gene expression. Infect. Immun. *56*, 2967-2971.

165. Poprawski, T. J., and Yule, W. N. (1990). Bacterial pathogens of *Phyllophaga* spp. (Col., Scarabaeidae) in southern Quebec, Canada. J. Appl. Entomol. *109*, 414-422.

166. Pouwels, P. H., Enger-Valk, B. E., and Brammar, W. J. (1985). Cloning vectors. (New York: Elsevier).

167. Rutter, J. M. (1988). Bacterial toxins as virulence determinants of veterinary pathogens: an overview. In Virulence mechanisms of bacterial pathogens, J. A. Roth, ed. (Washington,D.C.: American Society of Microbiology), pp. 213-227.

168. Ruvkun, G. B., Sundaresan, V. Ausubel, F. M. (1982) Directed transposon Tn5 mutagenesis and complementation of *Rhizobium meliloti* symbiotic nitrogen fixation genes. Cell *29*, 551-559.

169. Rydén, A. C., and Holfsten, B. (1968). Some properties of the extracellular proteinase and the cell-bound peptidase of *Serratia*. Acta Chem. Scandinav. *22*, 2803-2808.

170. Saier, M. H., Schmidt, M. R., and Leibowitz, M. (1978). Cyclic AMP-dependent synthesis of fimbriae in *Salmonella typhimurium*: Effects of *cya* and *pts* mutations. J. Bacteriol. *134*, 356-358.

171. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). Molecular cloning. A laboratory manual, 2nd ed. (New York: Cold Spring Harbor Laboratory Press).

172. Sanderson, K. E., and Hurley, J. A. (1987). Linkage map of *Salmonella typhimurium*. In *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaecter, and H. E. Umbarger, eds. (Washington, D.C.: American Society for Microbiology), pp. 877-918.

173. Sanger, F., Nicklen, S., and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA *74*, 5463-5467.

174. Schaffer, S., Hantke, K., and Braun, V. (1985). Nucleotide sequence of the iron regulatory gene *fur*. Mol. Gen. Genet. *200*, 110-113.

175. Schmitz, G., and Braun, V. (1985). Cell-bound and secreted proteases of *Serratia marcescens*. J. Bacteriol. *161*, 1002-1009.

176. Schmoll, T., Boudega, M. O. H., and Hacker, J. (1990). Use of wild type gene fusion to determine the influence of environmental conditions on expression of the S fimbrial adhesion in an *Escherichia coli* pathogen. J. Bacteriol. *172*, 5103-5177.

177. Schneider, D. R., and Parker, C. D. (1978). Isolation and characterization of protease-deficient mutants of *Vibrio cholerae*. J. Infect. Dis. *138*, 143-151.

178. Shum, T. (1992). Molecular analysis of a chitinase locus in *Serratia entomophila* [Masters of Science Thesis]. (University of Canterbury, Christchurch, New Zealand).

179. Slatten, B. H., and Larson, A. D. (1967). Mechanism of pathogenicity of *Serratia marcescens*. I. Virulence for the adult boll weevil. J. Invertebr. Pathol. *9*, 78-81.

180. Slauch, J., and Silhavi, T. J. (1991). Genetic fusions as experimental tools. Methods Enzymol. *204*, 213-248.

181. Smyth, C. J. (1982). Two mannose-resistant haemagglutinins on enterotoxigenic *Escherichia coli* of serotype 06:K15:H16 or H-isolated from travellers' and infantile diarrhoea. J. Gen. Microbiol. *128*, 2081-2096.

182. Smyth, C. J., and Smith, S. G. J. (1992). Bacterial fimbriae: variation and regulatory mechanisms. In Molecular biology of bacterial infection, C. E. Hormache, C. W. Penn, and C. J. Smith, eds. (Cambridge: Cambridge, University Press), pp. 267-297.

183. Spangler, B. D. (1992). Structure and function of cholera toxin and the related *Escherichia coli* heat-labile enterotoxin. Microbiol. Rev. *56*, 622-647.

184. Staskawicz, B., Dahlbeck, D., Keen, N., and Napoli, C. (1987). Molecular characterization of cloned avirulence genes from race 0 and race 1 of *Pseudomonas syringae* pv. *glycinea*. J. Bacteriol. *169*, 5789-5794.

185. Steinhause, E. A. (1959). *Serratia marcescensi*. Bizio as an insect pathogen. Hilgardia *28*, 351-380.

186. Stucki, G., Jackson, T. A., and Noonan, M. J. (1984). Isolation and characterization of *Serratia* strains pathogenic for larvae of the New Zealand grass grub *Costelytra zealandica*. New Zealand J. Sci. *27*, 255-260.

187. Stüber, D., and Bujard, H. (1981). Organization of transcriptional signal in plasmids pBR322 and pACYC184. Proc. Natl. Acad. Sci. USA *78*, 167-171.

188. Tamura, M., Nogimori, K., Murai, S., Yajima, M., Ito, K., Katada, T., Ui, M., and Ishii, S. (1982). Subunit structure of islet-activating protein, pertussis toxin, in conformity with the A-B model. Biochem. *21*, 5516-5522.

189. Tashiro, H., and Steinkraus, K. H. (1966). Virulence of species and strains of milky disease bacteria in the European chafer *Amphimallon majais* (Razzoumowsky). J. Invertebr. Pathol. *8*, 382-389.

190. Taylor, R. K., Manoil, C., and Mekalanos, J. (1990). Broad-host-range vectors

for delivery of Tn*phoA*: use in genetic analysis of secreted virulence determinants of *Vibrio cholerae*. J. Bacteriol. *171*, 1870-1878.

191. Taylor, R. K., Miller, V. L., Furlog, D. B., and Mekalanos, J. J. (1987). Use of *phoA* gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. Proc. Natl. Acad. Sci. USA *84*, 2833-2837.

192. Traub, W. H. (1980) Bacteriocin and phage typing of *Serratia*. In *Serratia* genus, A. V. Graevenitz and S. J. Rubin, eds. (CRC Press Inc., Bocaraton, Florida) pp. 80-100.

193. Trought, T. E. T., Jackson, T. A., and French, R. A. (1982). Incidence and transmission of disease of grass grub (*Costelytra zealandica*) in Canterbury. New Zealand J. Exp. Agric. *10*, 79-82.

194. Uhlin, B. E., Båga, M., Göransson, M., Lindberg, F. P., Lund, B., Norgren, M., and Normak, S. (1985). Genes determining adhesin production in uropathogenic *Escherichia coli*. Curr. Top. Microbiol. Immun. *118*, 163-178.

195. Upadhyaya, N. M., Glare, T. R., and Mahanty, H. K. (1992). Identification of a *Serratia entomophila* genetic locus required for amber disease in New Zealand grass grub (*Costelytra zealandica*). J. Bacteriol. *174*, 1020-1028.

196. Van Heyningen, W. E. (1955). The role of toxins in pathology. In Mechanism of microbial pathogenicity, J. W. Howie, and aj O'Hea, eds. (Cambridge: Cambridge University Press), pp. 17-37.

197. Vasil, M. L., Kabat, D., and Iglewski, B. H. (1977). Structure activity relationships of an exotoxin of *Pseudomonas aeruginosa*. Infect. Immun. *16*, 353-361.

198. Vasil, M. L., Holmes, R. K., and Finkelstein, R. A. (1975). Conjugal transfer of a chromosomal gene determining production of the enterotoxin in *Vibrio cholerae*. Science *187*, 849-850.

199. Visser, B., Munsterman, E., Stoker, A. and Dirkese, W. G. (1990). A novel *Bacillus thuringiensis* gene encoding a *Spodoptera exigua*-specific crystal protein. J. Bacteriol. *172*:6783-6788.

200. Vieira, J., and Messing, J. (1987). Production of a single-stranded plasmid DNA. Methods Enzymol. *153*, 3-11.

201. VonHeijne, G. (1986). A new method for predicting signal sequences cleavage sites. Nucl. Acids. Res. *14*, 4683-4690.

202. Weiss, A. A., and Hewlett, E. C. (1986). Virulence factors of *Bordetella pertussis*. Ann. Rev. Microbiol. *40*, 661-686.

203. Wigley, P., and Chilcott, C. (1990). *Bacillus thuringiensis* isolates active against the New Zealand pasture pest *Costelytra zealandica* (Coleoptera:Scarabaeidae). Abstracts of the Vth International Colloquium on Invertebrate Pathology and Microbial Control. Adelaide, Australia, 20-24 August, p 344.

204. Wilson, C. (1988). Intrinsic factors of the pathogenicity of *Serratia entomophila* toward the New Zealand grass grub *Costelytra zealandica* (White) [masters of science Thesis]. (University of Canterbury, Christchurch, New Zealand).

205. Wilson, C. J., Mahanty, H. K., and Jackson, T. A. (1992). Adhesion of bacteria *Serratia* spp. to the foregut of grass grub *Costelytra zealandica* (White) and its relationship to the development of amber disease. Biocont. Sci. Tech. *2*, 59-64.

206. Wretlind, B., Sjoberg, L., and Wadstrom, T. (1977). Protease-deficient mutants of *Pseudomonas aeruginosa*: pleiotropic changes in activity of other extracellular enzymes. J. Gen. Microbiol. *103*, 329-336.

207. Wright, A. C., Morris, J. G., Maneval, D. R., Richardson, K., and Kaper, J. B. (1985). Cloning of the cytotoxin-hemolysin gene of *Vibrio vulnificus*. Infect. Immun. *50*, 922-924.

208. Yamakawa, Y., and Ohsaka, A. (1977). Purification and some properties of phospholipase C (α-toxin) of *Clostridium perfringens*. J. Biochem. *81*, 115-126.

209. Yamamoto, T., Ariyoshi, A., and Amako, K. (1985). Fimbria-mediated adherence of *Serratia marcescens* US5 strain to human urinary bladder surface. Microbiol. Immunol. *29*, 677-681.

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